IN VITRO AND IN VIVO STABILITY AND METABOLISM OF NEUROPEPTIDES AND THEIR BRAIN-TARGETED PRECURSORS: METHOD DEVELOPMENT AND APPLICATIONS

BY

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TO MY FATHER

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In evaluation of the in vitro approach, peptide degradation in brain homogenate and metabolism of synthetic dynorphins have been studied. The in vitro approach was suitable for measuring the stability and obtaining kinetic data (k, $t_{1/2}$). However, detection of a full set of metabolites formed during degradation could not be done for most of the peptides studied due to tissue contamination.

For the in vivo approach for studying peptide metabolism, microdialysis conditions have been set for delivering the required amount of peptide to the tissue and recovering the metabolites and background-free microdialysates.

Upon investigating the CDS approach based on sequential metabolism for peptides

(enkephalin analogs and TRH), the release of biologically active peptides from brain-targeted conjugate peptides by specific peptidases has been successfully confirmed via in vivo and in vitro techniques.

Upon investigating the CDS approach based on sequential metabolism for peptides (enkephalin analogs and TRH), the release of biologically active peptides from brain-targeted conjugate peptides by specific peptidases has been successfully confirmed via in vivo and in vitro techniques.

For conjugates with spacers intended to be cleaved by prolyl endopeptidase (PP, PA, AP and AA), half-lives were one to two orders of magnitude shorter than those of the dipeptidyl peptidase degradation. Distribution of prolyl endopeptidase activity within the brain was also investigated. Among fractions incubated with T^{+}-Pro-Pro-DADLE, the rate of reaction in the medulla oblongata area was about 18 times slower than that in cortex area.

These results indicate the unequal regional distribution of PPCE (post-prolyl cleaving enzyme) activity against T^{+}-Pro-Pro-DADLE which results in the formation of DADLE, an opioid peptide analog.

In summary, complementary in vitro and in vivo techniques were developed to study the stability and metabolism of peptides in the CNS. These techniques were used to evaluate peptidase cleavages involved in the sequential metabolism that results in the CNS-targeting of pharmacologically active peptides. The peptidase cleavages were found

to yield the target molecules, but the rates of reaction were varied in wide range depending on the prevailing peptidase and on the substrate properties. The apparent hypothesis was that the reaction rate in the step involving the peptide release influence the pharmacological action according to a U-shaped curve; rapid cleavage could shorten the duration of action significantly, and slow release at the site of action could result in a peptide concentration below the therapeutic levels.

CHAPTER 1

INTRODUCTION

One of the most important processes of brain-targeting by molecular packaging and sequential metabolism (Bodor et al.,1992; Prokai et al., 1994) is the peptide release which relies on specific brain peptidases. So far, limited information has been available on the mechanistic and kinetic aspects of the reactions catalyzed by these enzymes for the specific conjugates involved as substrates.

The objectives of this research were to develop and evaluate the stability and metabolism of peptides, their analogs and derivatives in the brain, as well as to use these methods to study specific peptide derivatives that allow for predictable and controlled extracellular activation to yield phamacologically active compounds at the site of action after brain targeting by molecular packaging and sequential metabolism. Studies were focused on selected opioid peptides and thyrotropin-releasing hormone. In addition to in vitro models, a comprehensive in vivo monitoring system has been developed to analyze the metabolism of peptides in brain tissues of experimental animals. In order to circumvent the problems associated with an excised tissue, the novel tool of microdialysis sampling of living tissue was used. This technique is

uniquely suited for the elucidation of the integrated biochemistry of neuropeptides in the intact brain.

This dissertation, after overviewing the theoretical background and previous studies in this field (Chapter 2), decribes the experimental approaches to investigate stability and metabolism of peptides in the brain (Chapter 3), and evaluation and applications for the selected substrates (Chapter 4).

CHAPTER 2

REVIEW OF LITERATURE

Neuropeptides

The discovery of a wide range of biologically active peptides (adrenocorticortropin, α-endorphin, β-endorphin, bradykinin, dynorphin, gastrin, growth hormone, insulin, kyotorphin, leu-enkephalin, met- enkephalin, neurotensin, secretin, substance P, thyroid-releasing hormone, vasopressin, etc.) in the nervous system and growing knowledge of their characteristics have contributed to new theories of information transfer and chemical integrative process in the brain (Bloom, 1979; Schmitt, 1984).

Neuropeptides are involved in the regulation of the major bodily homeostatic systems, have local effects on cerebral circulation and on neuronal firing, are involved in developmental structure-function relations and the acquisition of neuronal plasticity, and are implicated in behavioral expressions. They can act, regarding the distance over which they exert their effects, as neurotransmitters (or neuromodulators), neurohormones, paracrine mediators, or hormones. For example, for many years peptides have been known to exist within the CNS, but an awareness of the large number of peptides that serve as neurotransmitters is relatively recent.

The discovery in 1975 of the enkephalins and their role as endogenous ligands for opiate receptors initiated an explosion of neuropeptide research that continues to the present. It is now generally acknowledged that peptide transmitters exist throughout the CNS. Therefore, peptide neurotransmitters have varied functions and are involved in most CNS activities.

Specific neuropeptide functions can, however, be confined to the central nervous system, and loss of such functions may become manifested as neurological diseases.

Neuropeptides are, therefore, potential drugs for treating various central nervous system (CNS) diseases. The metabolic instability of peptides represents one of the challenges in using peptides as drugs, since there exists a whole class of enzymes bent on their destruction--the peptidases. The peptide can be rapidly inactivated by ubiquitous peptidases prior to its delivery to the site of action and also at the site of action. Potential peptide and protein drugs are subject to degradation by numerous enzymes or enzyme systems throughout the body. Hydrolytic cleavage of peptide bonds by protease, such as enkephalinases, and chemical modification of the peptides or proteins, such as oxidation and phosphorylation, are happened. Hydrolysis is by far more common. Therefore, a major challenge in peptide drug development is to overcome the enzymatic barrier that limits the duration of action for peptide drugs. Degradation usually begins at the site of administration and can be extensive. The enzymatic barrier has three essential features, as follows.

First, since proteases and other proteolytic enzymes are ubiquitous, peptides and proteins are usually susceptible to degradation in multiple sites, including the site

of administration, blood, liver, kidney and vascular endothelia, etc. Consequently, peptides and proteins must be protected against degradation in more than one anatomical site for them to reach their target sites intact.

Second, almost all the peptidases and proteases may be capable of degrading a given peptide (Palmieri and Ward, 1983; Ward, 1984; Palmieri et al., 1985). The implication is that protecting a peptide or protein from degradation by one protease/peptidase may not necessarily lead to marked increase in its stability or in the amount of peptide/protein reaching its site of action (Dodda Kashi and Lee, 1986).

Third, a given peptide may be susceptible to degradation at more than one linkage within the molecular backbone; each locus of hydrolysis is mediated by a certain peptidase/protease. Often, even when one linkage is modified to circumvent one peptidase/protease, the rest of the peptide molecule is still vulnerable to other peptidase/protease. This usually manifests itself as a shift in the relative proportion of the various degradation products of a given peptide.

Proteases and peptidase are essentially hydrolases; hence, they have the ability to cleave peptide bonds with the addition of water. Peptidase/protease rarely show absolute specificity in their action; hence, any protease has the potential to hydrolyze more than one substrate (Given et al. 1985).

Clearly, in order to promote the delivery of peptides from any route of administration, the many components of the enzymatic barrier must be controlled. This can be achieved to some extent by modifying the peptide or protein structure, through co-administration of protease inhibitors, or by using the formulation approach this problem. Also, the existence of blood-brain barrier is the major obstacle for the

development of pharmaceutically useful neuropeptides Most often, metabolically stable peptide analogues are usually developed to circumvent this problem. Also, the existence of blood-brain barrier is a major obstacle for the development of pharmaceutically useful neuropeptides.

The Blood-Brain Barrier

The blood-brain barrier (BBB) is a membranous barrier that segregates brain interstitial fluid from the circulating blood and is highly resistant to solute free diffusion. The BBB is comprised of two plasma membranes in series, the lumental and antilumental membranes of the brain capillary endothelium, which are separated by about 0.3 μm of endothelial cytosol. The capillary endothelium in vertebrate brain and spinal cord is endowed with unique anatomic specialization called tight junctions that act as a zipper to close the interendothelial pores that normally exist in microvascular endothelial barriers in peripheral tissues. (Brightman, 1977)

The angioarchitecture of the human brain is based on the vascular cerebellar cortex. The tubular capillaries are formed by the endothelial cells folding over to form a lumen of approximately 6 μm in diameter. The capillaries are approximately 40 μm apart. Since it takes a small molecule such as glucose approximately 2 seconds to diffuse 40 μm, and since the capillary transit time in the brain, i.e., the time it takes for plasma to completely travel through brain, is about 1 second, the angioarchitecture of the brain allows for the solute equilibration throughout the brain interstitial space once the circulating molecule traverses the limiting endothelial barrier. The cerebral

microvasculature differs in several important respects from those capillaries present in the periphery (Fig 2-1).

First, the endothelial cells which comprise the cerebral microvessels are tightly joined to one another. This unique architecture prevents the bulk movement of materials between cells and forces compounds to diffuse directly through the phospholipid cells membrane if they are to gain access to the brain parenchyma. Since only those agents with sufficient affinity for the lipid membranes will penetrate the BBB, hydrophilic molecules, including many drugs, are excluded (Levin, 1980). Other distinguishing features of cerebral capillaries are that they are not penetrated and that they maintain a vesicular transport system of relatively low activity. These functions substantially restrict generalized movement of materials into the CNS and have clearly evolved to protect the delicate environment necessary for optimal neural functioning.

Histochemically, the BBB is also distinct from peripheral capillary systems in that high concentrations of various lytic enzymes are present. These protein catalysts include catechol-O-methyl transferase, monoamine oxidase, γ-aminobutyric acid transaminase, enkephalinase and various others (Levin, 1977). This enzymatic barrier prevents the uptake of blood-borne neurotransmitters and neuromodulators and, again, acts to isolate the CNS from potential neurochemical disruption.

While the barrier properties are an integral component of the BBB, they incompletely describe the system. Various hydrophilic nutrients such as glucose and metabolic wastes such as lactate must be taken up or expelled. These compounds do not, however, easily penetrate the lipid barrier system. This paradox is explained by the

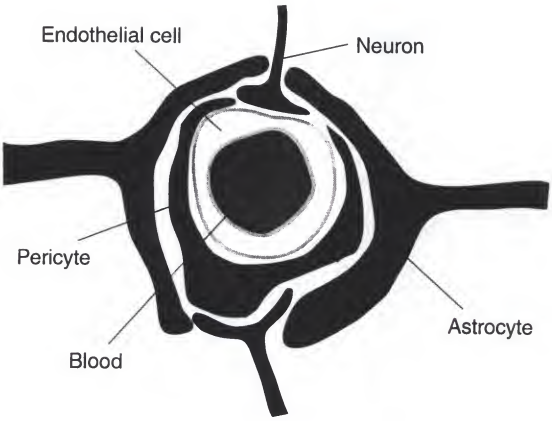

Fig 2-1. Model for cerebral microcirculation. The capillary endothelium is endowed with tight junctions, endothelial cells share microvascular basement membrane with pericytes, and more than 90% of capillaries are invested with astrocyte foot processes. Neuronal endings also innervate brain endothelial cells directly (Adapted from Pardridge, 1991)

presence of specific carrier systems in cerebral capillaries which ferry the agents into and out of the brain. Unfortunately, while these systems efficiently interact with sugars, amino acid, nucleotides and nucleotide bases, etc., they are, for the most part, not involved with drug transport.

Thus, the BBB acts to isolate and protect the CNS from periodic peripheral changes which might disturb neurofunctioning. This barrier system also restricts the movement of many potentially important drugs or hormones making the treatment of cerebral disease difficult, if not impossible.

Transport of peptide molecules across the BBB cannot be ruled out, but it is unlikely that endogenous peptides pass the BBB in physiologically significant amounts. Within the circumventricular organs (CVOs), the peptide molecules actually reach the cellular elements of the tissue. There is, however, no evidence of penetration to deeper layers. Most of the naturally occurring neuropeptides are hydrophilic and, thus, do not cross the BBB in the absence of a specific transport system in the BBB. Carrier-mediated transport of several dipeptides and tripeptides occurs in the brain. Certain large peptides (IGF-I, IGF-II, insulin, transferrin, and cationized albumin) are known to have receptors in the BBB. These receptors are identified on the luminal surface of the brain capillaries and are believed to act as transcytosis systems, since they are expected to be present in both luminal and antiluminall borders. Recent studies (Pardridge, 1986) have indicated that Leu-enkephalin is also taken up intact at the luminal side of the BBB. However, it does not rule out the possibility that it may be metabolized during the next steps in one of the compartments in parallel. These compartments may involve the cytosolic endothelial space, luminal surface of the

BBB, glial-end foot layer in apposition with the antiluminal side of the capillary endothelium, and/or enkephalinergic synaptic regions juxtaposed to the brain microvessels. The crucial characteristic of an active (carrier-mediated) transport system is its saturability.

Strategies of Peptide Delivery to the Brain

Various strategies are available for directing centrally active peptides into the brain (Pardridge, 1991; Prokai, 1996 and 1997). They can be grouped into three categories: (i) invasive procedures, (ii) physiological based strategies and (iii) pharmacological based strategies. Invasive strategies include implantation of an intraventicular catheter, followed by pharmaceutical infusion into the ventricular compartment. Due to the deep convexities of the human brain surface, most regions of the brain are no more than 0.5-1.0 cm away from the ependymal surface or the cortical surface of the CSF compartment. Taking an average diffusion coefficient (2×10^{-6} cm^2s^{-1}) for a peptide of 2,000-5,000 molecular weight, the effective diffusion distance of the substance is substantially less than 1 mm, if the degradation rate is rapid ($t_{1/2} <$ 10 min). Therefore, the intrathecal administration of peptides would be expected to allow for delivery of the compound only to the surface of the brain (Poplack et al., 1981).

Another invasive method relies on the effect by an intracarotid infusion of a high concentration (>1 M) of osmotically active substances such as mannitol or arabinose. Their high local concentrations cause shrinkage of the brain capillary endothelial cells, resulting in the transient opening of the tight junctions (Neuwelt and

Kroll, 1994) which may facilitate the transport of molecules that otherwise cannot cross the BBB. The considerable toxic effects of the procedure should be taken into account, which can lead to inflammation, encephalitis, ect. and to seizures (as high as 20 % of the applications). On the other hand, an indiscriminate delivery occurs. In conclusion, invasive procedures are only justified for life-threatening conditions, but these surgical routes are not acceptable for less dramatic illnesses.

In physiologically based strategies to deliver into the CNS, the observed paucity of endocytotic activity of the brain capillary endothelium does not reflect the complete lack of various transcytosis mechanisms but rather their very low capacity. Physiologically based strategies involve the formation of chimeric peptides and rely on the existence of transcytosis systems for the transport of various large peptides and proteins through the BBB. Covalent coupling (for example, disulfide bond) of a peptide is not normally transported through the BBB to peptide or protein "vectors" which undergo receptor-mediated or adsorptive-mediated transcytosis. These so-called chimeric peptides may then be transported via these vectors through the BBB into the brain. After entering the interstitial space of the brain, the active peptide is released and it may then interact with the corresponding receptor to initiate pharmacological action in the brain. Design considerations in the development of effective chimeric peptides include vector specificity for the brain, vector pharmacokinetics, coupling between vector and peptide, cleavability of the vector-peptide linkage, and intrinsic receptor affinity for the peptide released from the transport vector.

Receptor ligands such as insulin, insulin-like growth factor and transferrin are often semi-tissue specific. The affinity of the chimeric peptide for the receptor is

invariably 1 to 2 log orders lower than it is for the native peptide, which may result in limited endocytosis of the chimeric peptide (Raso and Basala, 1984). Uptake by nonneural cells or by cells outside the CNS has also been found. To improve vector pharmacokinetics, adsorptive-mediated transcytosis of cationized albumin, whose capacity is far greater than that of receptor-mediated endocytosis (Kumagai et al., 1987), has been utilized in the transport of appropriate chimeric peptides through the BBB (Pardridge et al., 1990). Recently, anti-transferrin (OX26) and anti-insulin (Mab83-7 and Mab83-14) receptor antibodies have been proposed as efficient and selective BBB transport vectors (Pardridge et al., 1990). Receptor-specific antibodies are considered to be better delivery vectors than cationic proteins because they have higher BBB permeability (P)-surface (S) products (PS).

The coupling of therapeutic peptides to transport vectors has been achieved by covalent conjugation targeting surface amino, carboxyl, or thiol groups in proteins, or by high-affinity noncovalent binding of the biotinylated peptide to a covalent avidin-vector conjugate (Bickel et al, 1993). Covalent conjugation of nerve growth factor (NGF) to a vector (OX26) increased the delivery of NGF to the brain parenchyma from about 0.1% of the injected dose when free NGF was administrated to about 0.4% after intravenous injection of an equivalent amount of OX26-NGF conjugate (Friden et al.,1993). Cross-linkage reactions may, however, proceed with unsatisfactory (10-15%) yields.

In the avidin-biotin method, neutral avidin is covalently attached to the vector, for example by a thiol ether linkage to the ε-amino groups of lysine residues of the protein. Avidin is an avian protein that binds biotin with extremely high affinity (a

dissociation constant of about 10^{-15} M and a half-life of 89 days) (Green, 1990). Then, the biotinylated agent is used to attach the therapeutic molecule to the avidin-vector conjugate. The target therapeutic peptide must be monobiotinylated to prevent the formation of high molecular weight aggregates due to multiple binding sites for biotin in avidin. CNS activity has been demonstrated in rats by the increase of cerebral blood flow and cerebral blood volume after systemic administration (10 min intracarotid infusion) of a biotinylated vasoactive intestinal peptide (VIP) analog conjugated to an OX26-avidin vector. However, the fraction of administered dose of the chimeric peptide entering the brain tissue beyond the BBB was 0.256%/g brain, which was a moderate increase from the 0.096%/g achieved by intracarotid infusion of the avidin complex of biotinylated VIP analog (Friden et al.,1993).

Some serious drawbacks may arise from a physiologically based approach. The affinity of the chimeric peptide for the receptor is lower than that of the native peptide, which results in arrival in the rat brain at maximum 0.4% injected dose (Bickel et al., 1993).

Carrier- or receptor-mediated cellular transport has physiologically limited transport capacity (saturable) which might prevent pharmacologically significant amounts of most peptides from entering the brain. The low amounts delivered to the CNS stresses the need for extremely potent therapeutic peptides, a need which has not been met by specifically developed, CNS-cleavable chimeric peptides with pharmacological activity at or above 100 pmol on intracerebroventricular injection (Bickel et al., 1995). Other strict requirements for coupling of a peptide to an avidin-antibody vector, such as monobiotinylation, in vivo cleavability of the linker and

retention of biological activity after release, make this method very restrictive. Already discovered and potentially useful peptides may have to be radically resigned to enable them to be incorporated into chimeric peptides for potential delivery to the CNS by a physiologically based strategy.

The poor stoichiometry of the neuropeptide to the carrier molecule limits the mass transport of the target peptide.

Hydrophobic substances, because of their high lipid solubility, can generally diffuse freely across the BBB. Pharmacologically based strategies propose to turn the peptide lipid-soluble by chemical modification to permit this transport to take place. Peptide lipidization by forming diketopiperozines, e.g., from the C-terminal dipeptide of TRH, has been applicable only to small peptides (Friden et al., 1993).

Lipophilic peptide derivatives such as prodrugs (Bundgaard, 1992) are often designed to limit metabolism of the parent molecule (Moss, 1995). Prodrugs are inactive molecules that undergo (a single) metabolic or chemical conversion to the active drug in vivo. Peptide prodrugs are usually designed to increase lipid solubility and/or metabolic stability (Bundgaard, 1992). Lipid-soluble peptide prodrugs that can cross the BBB can only sustain active concentrations in the CNS if their blood concentrations are maintained at adequately high levels.

The chemical delivery system (CDS) is defined as a biologically inert molecule requiring several steps for conversion to the active drug, thereby enhancing drug delivery to the target site (Bodor, 1987). This strategy is distinct from the simple pharmacologically based approach in which a lipophilic peptide prodrug is applied.

There are three criteria for a brain-targeting CDS. First, it should be lipophilic enough to allow for brain uptake. Second, after the brain penetration, retention of the lipophilic molecule is required to prevent its efflux from the CNS. Third, the conversion intermediate should be degraded enzymatically according to a designed route to release the active molecule over a long period. The brain-targeting CDS has been extensively applied to various neurotransmitters and other pharmaceuticals. Among those, peptides are included as a major consideration. Since both the BBB and enzymatic degradation prevent the passage of the peptides from the general circulation to the brain tissue, the CDS (an enzyme-based strategy) is an excellent candidate for peptide brain-delivery.

A possible approach is the derivatization of the peptide to produce a transport form that is markedly more lipophilic than the parent peptide as well as resistant to the various peptidases. Yet, it must remain cleavable by enzyme-catalyzed hydrolysis at a prescribed joint to have the sustained brain-specific release of the parent peptide in situ.

The efficacy of this kind of peptide delivery system depends not only on the effective lipophilicity of the molecular package, but also minimal exposure of the target peptide to vascular peptidases. Therefore, the CDS approach seems to be an excellent candidate for brain targeted delivery of enkephalin (Bodor et al., 1992; Prokai-Tatrai et al., 1996) and other peptides (Prokai et al., 1994).

Brain-Targeting of Neuropeptides by Molecular Packing and Sequential Metabolism

Brain-targeting of Leucine-enkephalin analogs

Opioids, by definition, act on the opioid receptors in the brain. The naturally occurring compounds that operate on these receptors (endogenous opioids) are peptides: enkephalins, dynorphins, and endorphins (Hughes, 1983). All three classes are closely related structures and have a degree of flexibility that allows them to take up different conformations. Many opiates are useful and medically important narcotic analgesics. Major opiates include natural substances, such as opium, morphine, and codeine: semisynthetic drugs produced by minor chemical alteration of the poppy product, (heroin, hydromorphone and oxycodone), and synthetic analgesics, such as propoxyphene and mepedrine. As a group, these drugs are liable to misuse (Schick and Schudziarra, 1985). These substances are very addictive, and physical dependence develops after relatively short-term use. Tolerance also develops rapidly to most opiates, particularly to the most potent analgesics.

Morphine-like drugs produce analgesia, drowsiness, changes in mood, and mental clouding, etc. A significant feature of the analgesia is that it occurs without loss of consciousness. The relief of pain by morphine-like opioids is relatively selective, in that other sensory modalities are not affected. Continuous dull pain is relieved more effectively than sharp intermittent pain, but with sufficient amounts of morphine, it is possible to relieve the severe pain such as those associated with renal or biliary colic.

Opioid-induced analgesia is due to actions at several sites within the CNS and involves several systems of neurotransmitters. Although opioids do not alter the threshold or responsibility of afferent nerve ending to noxious stimulation or impair the conduction of the nerve impulse along peripheral nerves, they may decrease conduction of impulses of primary afferent fibers when they enter the spinal cord and decrease activity in other sensory endings. There are opioid binding sites (μ receptors) on the terminal axons of primary afferent within laminae I and II (substantia gelatinosa) of the spinal cord and in the spinal nucleus of the terminal nerve. Morphine-like drugs acting at this site are thought to decrease the release of neurotransmitter, such as substance P, that mediates transmission of pain impulses.

Other than analgesia, morphine can produce nausea, vomiting, feeling of drowsiness, inability to concentrate, difficulty in mentation, apathy, lessened physical activity, reduced visual acuity, body warmth, relief of stress, and euphoria (Duggan and North, 1983; Martin, 1983)

Morphine was isolated in 1805 by Serturner, its chemical structure was first correctly established in 1925 by Robinson and Gulland. The proof of the existence of an opiate receptor was accomplished in 1970, leading to the receptor mediated pain suppression in the brain as we know it by Goldstein. This stimulated the search for endogenous agonists, since it was hard to believe that all vertebrates had a receptor just for herbal and synthetic agonists. As a result of these efforts the structure of the enkephalins was published by Hughes et al. (1975) after their isolation from the pig brain.

Soon afterward, it was shown that they were degraded rapidly in mammalian serum (Hughes et al., 1975). This was followed by findings of methionine enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) in human plasma; consequently, new physiological roles were attached to them.

The isolation of the other two families of opioid peptides, the β-endorphins and the dynorphins, was also accomplished in 1975 (Bradbury et al., 1975; Cox et al., 1975).

Each family of opioid peptides is derived from a genetically distinctive precursor polypeptide and has a characteristic anatomical distribution. These precursors are now commonly designated as proenkephalin (also proenkephalin A), pro-opiomelancortin (POMC), and prodynorphin (also proenkephalin B). Each of these precursors contains a number of biologically active peptides, both opioid and nonopioid, that have been detected in blood and various tissues. Proteolytic processing of the precursors generates the active peptides that are expressed via peptide receptor systems at the target cell -- the opioid peptide receptors.

The significance of these findings became clear, when a series of discoveries revealed that the enkephalins do not only act as a neurotransmitter in pain control, but also possess neurohormone activities. Recently, the enkephalins have been observed to play a role as signal messengers in the communication among the nervous, endocrine and immune systems. They have been proven to take part in the immune response, the regulation of blood pressure, food intake and behavioral processes such as mood. The enkephalins seem to serve as a communication signal between the physiological and psychological states of an animal. They are, however, not tonically active; that is,

under conditions of normal bodily functions (homostasis) they play a minor role. If homeostasis is disrupted by stress, as in a painful stress, their activation results in a spectrum of responses.

This results in an ability to bind to more than one type of opioid receptor and, thus, to function as either a short-acting neurotransmitter or a longer-acting neuronal or hormonal modulator. There are at least three distinct opioid receptor types, each of which has a different pharmacological profile, tissue distribution, and different binding properties. Mu receptors (μ) are the main sites of action of narcotic analgesics, delta receptors (δ) play an important role in nociception and probably limbic functions, and kappa receptors (κ) are involved in certain types of pain. (Sigma and eta receptors are also suggested, but they are either not true opioid receptors, or their significance is not known).

In summary, the endogenous opioids are part of an extensive neuronal network in both the central and peripheral nervous systems involving multiple ligands and multiple receptors. The endorphins, enkephalins, and dynorphins are opioid peptides derived from glycoprotein precursors; proopiomelanocortin (POMC), proenkephalin, and prodynorphin, respectively. These substances exhibit complex pharmacology and exert diverse physiological effects in mammals. Analgesia is, however, their best-recognized central effect (Frederickson, 1984; Akil et al., 1984).

Leucine-enkephalin analogs that show improved metabolic stability are the simplest peptides that can be used to exert analgesia after i.c.v. administration by CDS. CNS-delivery of these compounds by molecular packaging and sequential metabolism

has been studied (Bodor et al., 1992; Prokai-Tatrai et al., 1996), and many important characteristics of the modifying functional moieties have been revealed.

Stability studies and pharmacological investigations have shown the importance of stabilizing the peptide backbone (Bodor et al., 1992) and of selecting the appropriate spacer (Prokai-Tatrai et al., 1996). However, the release of the biologically active peptide has been only demonstrated in vitro, and stability studies have been done only for the limited number of spacer functions allowed by the CDS design.

The CDS employs interconversion of a lipophilic dihydropyridine moiety to a hydrophilic pyridium salt moiety that is an analog to the NADH <=> NAD^+ coenzyme system (Fig 2-2).

This lipophilic dihydropyridine carrier is covalently linked to an active drug to form the CDS that is able to penetrate the BBB. Upon systemic administration, the CDS can partition into several body compartments due to its enhanced lipophilicity. At this point, the CDS is simply working as a lipoidal prodrug. The carrier molecule is specially designed, however, to undergo an enzymatically mediated oxidation that converts the membrane-permeable dihydrotrigonellinate (the lipophilic dihydropyridine moiety) to a hydrophilic membrane impermeable, trigonellinate salt (the hydrophilic pyridium salt moiety) in vivo.

The polar carrier-drug conjugate is then trapped behind the BBB. Any of this oxidized salt that is present in the periphery will be rapidly lost as it is now an excellent candidate for elimination by kidney and liver. The conjugate that is trapped behind the BBB then slowly hydrolyzes to release the active drug in a sustained manner.

However, concentration of the active drug is low in the periphery, which minimizes dose-related side effects and toxicity. In addition, the active drug in the CNS is present mostly as an inactive conjugate, which offers two advantages which are lower central toxicity and increased dose interval.

For many smaller drugs, a simple redox targetor has proved to be applicable (Bodor and Brewster, 1983; Bodor and Simpkins, 1983). For peptides, however, the attachment of 1,4-dihydrotrigonelline to the NH_2-terminus alone will not furnish a sufficient increase in lipophilicity as well as be susceptible to cleavage by numerous exo- and endo- peptidases. In the strategy called "molecular packing," the peptide unit of the CDS appears only as a perturbation on the bulky molecule dominated by lipophilic modifying groups, that ensures the BBB penetration and confuses peptidases.

A chemical delivery system for peptide brain-delivery has been proposed based on all the considerations, as shown in Fig 2-3. A centrally active peptide sequence (P) is placed in a molecular package that disguises its peptide nature and provides biolable, lipophilic functions to penetrate the BBB by passive transport.

The design incorporates a 1,4-dihydrotrigonellinate targetor (T) at the NH_2-terminal of the peptide via a spacer (S) and a lipophilic ester (L) at the C-terminal. Because of the low amidase activity of the brain tissue, a spacer (S) is used to separate the peptide sequence (P) from the targetor part of the CDS. The spacer is selected based on the peptidolytic activity prevalent at the site of action, so that the release of the desired peptide is favored over the degradation induced by other peptidases.

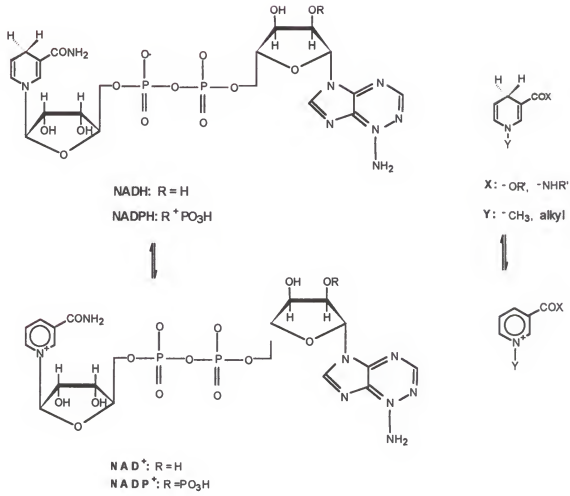

Fig 2-2. The NADH <=> NAD^+ coenzyme system and the trigonellinate <=> 1,4-dihydrotrigonellinate redox system

After the molecular package penetrates the BBB and enters the CNS, the 1,4-dihydrotrigonellinate targetor (T) undergoes an enzymatically mediated oxidation to become a hydrophilic, membrane impermeable trigonellinate salt (T^+) which traps the whole molecule behind the BBB and inside the CNS. Hydrolysis of this molecule provides a polar targetor-peptide conjugate which is a substrate for peptide degrading enzymes, such as dipeptidyl peptidases and post proline cleaving enzymes.

Through this sequential enzymatic degradation, the final biologically active peptide is released in a pharmacologically significant amount inside the brain. The peptide release is a critical step in this strategy. The spacer is used to assure a precise cleavage between the peptide and the targetor. Therefore, the spacer is the most important part of the peptide CDS which allows us to control pharmacological effects through the release of the biologically active peptide. The cleavage of the biologically active peptide should also be prevented.

For example, it has been shown that a targetor-spacer-peptide conjugate for the $[D\text{-}Ala]^2$-leucine enkephalin degrades without giving the biologically active peptide when there is L-Leucine at the C-terminus. Therefore, this CDS does not have a significant analgesic effect. This means that peptide release is not competitive with peptide degradation. With D-Leucine at the C-terminus, degradation of the peptide is prevented, and there is a slow release of the peptide analog. This gives sustained analgesia. In designing peptide CDSs, different spacers that may increase or decrease the endopeptidase can also be used. They cleave peptide after proline and alanine. This method was demonstrated with the Leu-TRH CDSs.

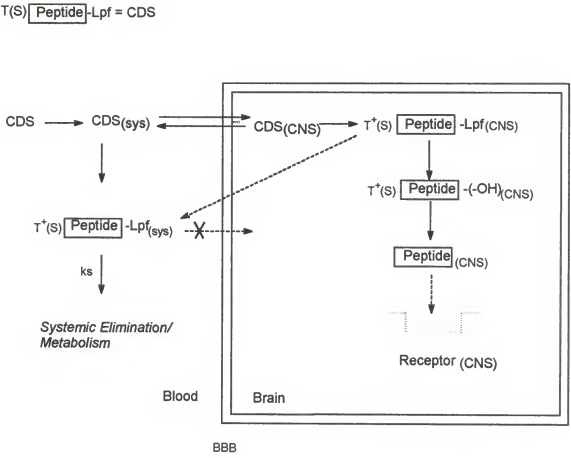

Fig 2-3. Brain targeting of peptides by sequencial metabolism

In summary, brain delivery occurs by the following sequence: (1) the CDS crosses the BBB by passive transport, and once in the CNS (2) the targetor is converted to a membrane impermeable form, and (3) the lipophilic function is removed to yield a relatively stable peptide conjugate that is retained in the CNS. Finally, (3) peptidases (previously dicussed in Chapter 2) process the conjugate to release the desired peptide and neuropeptide-specific enzymes may also be involved. This sequence needs the following enzymes:

(1) Oxidoreductases: Oxidoreductases (Chance and Williams, 1956) carry out the first step of sequential metabolism. This enzymatic reaction needs a -NAD^+ <=> NADH coenzyme- and prevents the peptide from leaving the central nervous system after delivery. The mechanism of this oxidation was extensively examined (Hoek and Rydstrom, 1988), and it was suggested to be analogous to the oxidation of NAD(P)H, a coenzyme associated with numerous oxidoreductases and cellular respiration. Various forms of a dihydropyridine-type targetor can be applied. However, the 1,4-dihydrotrigonellyl moiety was found to be the best.

(2) Esterase/Lipase; Esterase (Barman, 1969) and/or lipase enzyme (Dixon and Webb, 1964) are capable of removing the lipophilic ester (Lpf) part which is the second step of sequential metabolism after penetration through the BBB (the lipophilic function is removed to yield a relatively stable peptide conjugate that is retained in the CNS). The size/lipophilicity of the ester group (cholesteryl or 1-adamantaneethyl) for the efficacy of the CDSs to introduce CNS-mediated analgesia upon iv administration was evaluated for developing an effective synthesis strategy for obtaining peptide CDSs

(Prokai-Tatrai et al., 1996). By the test for analgesic efficacy based on tail-flick latency measurements in the rat, the selection of the lipophilic group can greatly influence the efficacy of the CDS. The size/lipophilicity of the Lpf is evidently a basic requirement for the delivery of the peptide through the BBB. Compounds with cholesteryl (Cho) on the C-terminus produced higher tail-flick latencies than those having 1-adamantaneethyl (Ada). Consequently, the size of Lpf itself has an important contribution to the successful delivery of peptide; the Lpf must provide a sufficient increase in lipid-solubility and protect the peptide from degradation during the transport to the CNS.

(3) Peptidases; The ultimate aim of the sequential enzymatic conversion is to release the biologically active peptide at the site of action. An important event in the sequence is the removal of the targetor and the attached spacer from the peptide. The design of this enzymatic cleavage should assure control of selectivity by allowing the reaction to take place at the desired chemical bond while the occurrence of parallel or side reactions is minimized. Peptidase cleavages usually fulfill the requirement for high selectivity; thus, spacer functions used thus far have relied on extending the peptide sequence with a single amino acid residue or a pair of amino acid residues. Due to the significance and specificity of Pro and Ala residues in neuropeptide processing (Schwartz et al., 1981), dipeptidyl peptidase II and IV, as well as prolyl endopeptidase were involved. The sequence of dipeptidyl peptidase II, dipeptidyl peptidase IV and prolyl endopeptidase is shown in the following Fig 2-4.

These enzymes usually have a preference for Pro at the cleavage site. Recent

Dipeptidyl peptidase II,

↓

Xaa-Pro-Yaa--- (Xaa = N-terminal amino acid residue,

↓

Xaa-Pro-Yaa--- may be replaced by trigonellyl)

Dipeptidyl peptidase IV

↓

Xaa-Pro-Yaa--- (Yaa ≠ Pro, Hyp)

↓

Xaa-Ala-Yaa--- (Yaa ≠ Pro, Hyp)

↓

Xaa-Hyp-Yaa--- (Yaa ≠ Pro, Hyp)

Prolyl Endopeptidase (EC 3.4.21.26)

(=Post-proline Cleaving Enzyme, Post-proline Endopeptidase, TRH-deamidase,

Brain-kinase B, Endooligopeptidase B)

↓

---Pro-Xaa--- (Xaa ≠ Pro)

[---Ala-Xaa---]

Fig. 2-4 Designed-in Enzymatic Cleavage for "Locked-in" Peptide Precursors

study has shown that Pro spacers may also be preferred in peptide CDSs (Prokai-Tatrai et al., 1996). Altogether peptidase cleavage may provide a convenient means for controlling the rate of peptide release from the CDS and, thus, the concentration of the biologically active peptide in the CNS, and its duration of action.

(4) Neuropeptide-Specific Enzymes: The C-terminal glycine functions as an amide donor for proline, due to an enzymatic activity designated peptidyl glycine α-amidating monooxygenase (PAM) which requires Cu^{2+}, ascorbic acid, and molecular oxygen (Bradbury et al., 1982; Husain and Tate, 1983). Glutamine (Gln) is the precursor of the N-terminal pyroglutaminyl (pGlu), and the cyclization is catalyzed by a specific enzyme, glutaminyl cyclase (Fischer and Spiess, 1987). These enzymes are necessary, respectively, when the peptide to be brain-targeted contain C-terminal carboxyamide or N-terminal pGlu (TRH analogs, such as pGlu-Leu-Pro-NH_2, need both PAM and glutaminyl cyclase that convert the Glu-Leu-Pro-Gly progenitor sequence to the target peptide).

Brain-targeting of thyrotrophin-releasing hormone analogs

Thyrotrophin-releasing hormone (TRH) is the hypothalamic releasing factor that was characterized, establishing the fundamental proof for the existence of a neuroendocrine regulation of pituitary functions by hypothalamic neuronal structure (Bõler et al., 1969). A variety of behavioral effects are induced by peripheral and central application of TRH, such as increased locomotor activity (Heal et al., 1983, Andrews and Sahgal, 1984, Webster et al., 1984). The well-known CNS effect of TRH is the analeptic action; reduction of pentobarbital narcosis or haloperidol-induced

catalepsy (Sharp et al., 1984). TRH and its analogues can also increase extracellular acetylcholine (AC) levels, accelerate AC turnover, improve memory and learning, and reverse the reduction in high-affinity choline uptake induced by lesions of the medial septal cholinergic neurons (Schmidt, 1977; Horita et al., 1989; Mellow et al., 1989; Yamamoto and Shimizu, 1987, 1988). Furthermore, TRH has been indicated to be beneficial for motor neuron diseases, spinal cord trauma (Faden et al., 1989) and Alzheimer's disease (Lampe et al., 1991; Griffiths, 1986, 1987). Several analogues of TRH have been synthesized to separate the endocrine (i.e. TSH-releasing activity) from the CNS effects, however, decreased or absent TSH-releasing activity (Szirtes et al., 1984).

The principles of the chemical delivery system (CDS) method have been adapted to other neuropeptides with free amino- and carboxyl-termini. However, there is no such type of termini in TRH and its analogues modified only at the His residue. Therefore, these peptides convey problems to the design of a targeting system due to the presence of the amino-terminal pGlu residue and of the prolinamide at the carboxyl-terminus. The solutions to these problems (Prokai et al., 1994) have been derived from considering the biosynthesis of TRH, which involves post-translational cleavage of a large precursor polyprotein synthesized on ribosomes (Jackson, 1989). Peptidyl glycine α-amidating monooxygenase (PAM) converts the C-terminal glycyl residue to amide (Bradbury et al., 1982; Husain and Tate, 1983). The final peptide is formed in part from a precursor, Gln-His-Pro-NH_2. Brain targeting of pro-TRH tetrapeptide with Gln and Gly amino- and carboxy-terminus, respectively, should result in the

formation of pGlu-His-Pro-NH_2 because of the apparent involvement of PAM and glutamyl cyclase (Fischer and Spiess, 1987).

It was postulated that TRH analogues modified at the second amino acid residue also could be obtained from a Gln-Xaa-Pro-Gly progenitor sequence (Xaa is an unspecified amino acid residue). Because $[Leu]^2$-TRH (pGlu-Leu-Pro-NH_2) and $[Nva]^2$-TRH were found to exert a 2.5-fold and 10-fold increase in CNS activity, respectively, and also show decreased TSH-releasing properties, as compared to TRH, CDSs have been designed, synthesized and evaluated for these CNS-active TRH analogues.

In this design, the peptide conjugate with the liberated Gly C-terminus produces the corresponding proline amide, followed by a cleavage that removes the targetor + spacer moiety releasing Gln-Leu-Pro-NH_2. The role of the spacer (S) function is to facilitate this cleavage via appropriate enzymes; dipeptidyl dipetidases (Johnson and Shah, 1978), or postproline cleavage enzyme (PPCE) (Kenny et al., 1976; Koida and Walter, 1976) that have specific cleaving sites (Pro or Ala) in a peptide sequence. To assess the significance of the spacer function to allow for maximizing the given pharmacological effect, the chemical targeting system containing the possible combinations of Pro and Ala as single- and double- residue spacers was synthesized and evaluated (Prokai et al., 1997).

Because of the limited knowledge about the selection criteria for the optimal spacer function and about the relative affinity of the possible spacer combinations to the specific enzymes, the focus of the study was only the investigation of the peptide release in the reaction sequence involved in the CDS approach.

The further evaluation of the CDS approach will require the selection of therapeutically useful peptides and the design/optimization of molecular strategies intended to achieve brain-enhanced delivery and sustained release at the site of action.

CHAPTER 3

MATERIALS AND METHODS

Experimental Methods of Studying Peptidase Activity in the Brain

It is critical in peptide drug development to determine which peptide candidates are enzymatically stable. Biostability studies have focused mainly on the issue whether the peptide maintains its integrity after intravenous (i.v.) administration (Powell, 1993). Therefore, in vitro degradation in serum and plasma is commonly incorporated into the methodology for measuring peptide biostability. Determination of the biostability in blood only is justified for peptides with intended systemic use. Metabolism of centrally acting neuropeptides in the brain is often a more critical process that limits their neuropharmaceutical efficacy than their systemic degradation. Breakdown of neuropeptides can alter their pharmacological profile, and their action also may be mediated through conversion to metabolites.

Brain homogenate (Pardridge,1991) or intact pieces of brain (Robsòn et al., 1983) from appropriate animal models can be used to evaluate the possible fate of neuropeptides in the CNS in vitro, because both soluble and membrane-bound peptidase will probably cleave neuropeptides. From in vitro studies, metabolites can

be identified and degradation kinetics can be obtained. However, in analysis of RPLC, there are considerably noisy from contaminants from the backgrounds, which causes the difficulty of identification of metabolites.

Homogenates, however, contain many cytosolic peptidases that the peptides may not be exposed to in an in vivo situation. The in vitro approaches has recently been complemented by in vivo techniques such as ventriculocisternal perfusion (Dixon and Traynor, 1990) and microdialysis (Molineaux and Ayala, 1990).

In vivo cerebral microdialysis produced samples that were practically free of interference by brain proteins, thus allowing the reliable identification of the peptide metabolites. Microdialysis for in vivo analysis has gained wide recognition as a sampling technique for the measurement of many endogenous neurochemicals and was first reported by Zetterstrom and Ungerstedt (1984). Conceptually, the method is based on the principle that compounds will diffuse down their concentration gradient, over a semipermeable membrane, into or out of the perfusion medium that flows through the microdialysis probe. The technique can be used to monitor the release of neurosubstance in the extracellular fluid of specific brain regions and eliminates the need to extract substances from post-mortem tissue (Zetterstrom and Ungerstedt, 1984). However, a disadvantage of the technique is that the dialysate is relatively dilute and contains high salt concentrations, conditions that normally give poor sensitivity when using many analytical techniques, including mass spectrometry.

In addition to the in vitro approach, this present study also deals with intracerebral microdialysis, an in vivo tool to monitor free concentrations of compounds in the brain of experimental animals. To date most of its applications have been in the area of neurochemistry. However, in pharmacokinetic and pharmacodynamic research the features of this technique are particularly useful.

Ideally, an in vivo method to be used for determination of the local pharmacokinetics of a drug in the brain should exhibit the following characteristics: (1) It should measure drug concentrations with high selectivity (i.e. there should be no interference from metabolites). (2) It should allow the measurement of free (unbound) concentrations.(3) It should allow the determination of local drug concentration and local concentration differences within the brain. (4) It should be possible to determine concentration versus time profiles within individual animals. (5) The technique should be non-invasive.

Quantitative autoradiography (QAR), imaging techniques, CSF sampling, in vivo voltammetry and intracerebral microdialysis that have been developed over the years fulfill these criteria only partially. Intracerebral microdialysis is based on measurements of drug concentrations in brain dialysate which supposedly reflect (but are not identical to) concentrations in brain extracellular fluid.

The most classical technique is push-pull perfusion (Phillippure, 1984) in which two concentric stainless steel tubes are placed directly into the brain and the ECF is perfused with damage at the tip of the cannula and relatively low recoveries from extracellular medium, while there is a need for 'clean up' of the perfusate before samples can be analyzed.

With the introduction of intracerebral microdialysis several improvements to this basic idea have been made. Intracerebral microdialysis involves the insertion of a microdialysis probe with a semipermeable part into a selected area of the brain. Substances around the semipermeable part of the probe will diffuse into or out of the perfusate, into the direction of the lowest concentration. Subsequently the dialysate can be collected for analysis.

The advantages of this technique are (1) the concentrations of compounds can be monitored in individual animals in a selected area of the brain; (2) dialysate concentrations reflect the free concentrations in the brain; (3) it is possible to use freely moving animals in the experiments; (4) the technique is relatively cheap; (5) the ex vivo samples permit analysis by virtually every technique and therefore allow selectivity; and (6) the dialysis principle provides samples that ate already free of proteins and other macromolecules, which evades clean-up procedures and eliminates potential ex vivo degradation of the substance of interest by enzymes.

Disadvantages in the use of this technique exist as well. These include: (1) the difficulty to estimate *in vivo* recovery of the compound of interest; (2) the diluting effect of dialysis, which causes the need for an analysis sensitive enough to detect small concentrations; and (3) the invasiveness of the technique obligates the investigator to be acquainted with the effects of trauma on the system under investigation in order to circumvent erroneous conclusions.

Neuropeptides are generally analyzed by immunological methods, such as radioimmunoassays (RIA). These methods afford high sensitivity (attomole to femtomole range) but lack complete specificity owing to cross-reactivity of the

antibody with substanced other than the specific peptide of interest. Early work by Caprioli and Lin (1990) and subsequently others, has demonstrated the technique of microdialysis coupled to mass spectrometry in studies involving drug metabolism in living animals. More recently, microdialysis/mass spectrometric techniques have successfully been used to follow endogenous release of neurotensin and methionine [Met^5]-enkephalin in the brain of unanesthetized freely moving rats.(Andren and Caprioli, 1995; Emmett and Caprioli, 1994).

Optimization and comparative evaluation of these techniques were done by using model peptides, most rigorously with Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu-OH). Preliminary studies have indicated that this small opioid peptide is metabolized predominantly by a single enzyme (aminopeptidase) in the extracellular region of the CNS. Therefore, results given by the methods used can be easily interpreted, and the experimental conditions can be optimized without involving complex kinetic equations.

Studies on dynorphins may be difficult, because they are highly unstable and rapidly metabolized. Breakdown of dynorphins can alter their pharmacological profile, and their action may be mediated through conversion to metabolites (Dixon and Traynor, 1990).

Because of the rapid breakdown of dynorphins, it is critical to examine their metabolism in brain tissue to correlate with their physiological role or pharmacological effects (Young et al., 1987). In rat membrane preparations, Dyn A (1-13) is rapidly degraded by membrane-bound exopeptidases (Cone and Goldstein, 1982) Although the involvement of various peptidases in the breakdown of

dynorphins in the central nervous system (CNS) also has been shown (Benter et al., 1990; Molineaux and Ayala, 1990; Persson et al., 1993) little is known about the overall CNS-metabolism of these peptides.

Cerebral microdialysis combined with mass spectrometry (Andren and Caprioli, 1995) is especially attractive for studying CNS metabolism of peptides, because it may enable continuous monitoring of biochemical events in brain extracellular tissue space in vivo and provide positive identification of the metabolites through the high molecular specifity of spectrometric method. Microbore LC was used off-line to separate the peptide metabolites for electrospray ionization (ESI) mass spectrometry, or a reversed-phase (C_{18}) packed fused-silica capillary was used for on-line desalting of the microdialysates prior to analysis. However, the peptides were eluted from the column with no or minimal chromatographic separation, and identification of the metabolites were done from a single (averaged) mass spectrum. ESI of peptides is characterized by multiple charging observed in the mass spectra reflecting the number of possible protonation sites in the molecules. Dynorphin peptides are highly cationic (due to multiple Arg and Lys residues), and doubly- and triply-charged ions are commonly obtained during ESI ionization (Dass et al., 1991) and complex peptide mixtures may produce complicated mass spectra.

To simplify assignment of mass spectra and possibly avoid time-consuming chromatographic separation, matrix-assisted laser desorption/ionazation (MALDI) mass spectrometry was evaluated as an alternative. MALDI is known to yield mostly singly-charged molecular ions for peptides and proteins.

As discussed in the previous chapter, brain peptidases assume a critical role in determining the success of the CDS approach to brain-targeting of peptides. Peptidases may prematurely cleave the sequence of the target peptide while it is still conjugated to the targetor (T^+) (Bodor et al., 1992). In addition, specific peptidases are involved in the release of the biologically active peptide from this conjugate according to the design. Optimization of the CDS strategy should, therefore, include efforts to minimize premature cleavage within the sequence of the target peptide, and also to facilitate or control the release of the biologically active molecule. Development of appropriate experimental techniques to evaluate these peptidase activities in the brain is essential to study the mechanism of CNS-targeting of peptides by CDSs and to further extend this strategy.

The metabolism of dynorphin A (1-8) by various regions of peptides in the rat brain (Dixon and Traynor, 1990), the amount of metabolite (Leu-enkephalin) of dynorphin A (1-8) were unevenly distributed. This means there are unevenly distributed enzyme activity, Therefore, this led us to undertake a study of prolyl endopeptidase activity for a leucine-enkephalin analogs conjugate by various regions in the rat brain.

Experimental Protocols

For in vitro studies, Sprague-Dawley rats (Av. wt. 250-300g) were killed and rat brain was collected and homogenized in 20 % w/w, artificial-CSF, pH 7.4,

then incubated at 37^0C after the addition of the peptide in 10-200 μM concentration. Samples (100 μl each) were removed periodically for analysis.

Reaction in each sample was stopped by blocking solution (5% acetic acid and 5% $ZnSO_4$ in H_2O, 100 μl) at various times. Then, mixtures were centrifuged at about 13,000g for 30 min. The supernatant was collected and (HPLC) analyses were done by microbore HPLC. Especially, after many trials and errors, the composition of this blocking solution was optimized. The procedure is summarized in Fig. 3-1.

HPLC calibration curves for determining concentrations were obtained by adding known amount of peptide into aliquots of brain homogenate transferred into ice-cold blocking solution and analyzing the supernatant after centrifugation.

Concentration-time profiles were analyzed by exponential fitting, assuming a pseudo-first order degradation. In vitro half-lives ($t_{1/2}$) were calculated from the rate constants (k) as 0.693/k.

In dissectional studies in the rat brain, Sprague-Dawley rats (Av. wt. 250-300g) were killed and the brain were carefully removed, blotted and chilled. Dissections were performed on an ice-cooled glass plate. Six regions were separated, and these will be described in text and shown in Fig 3-2 by the following simplified names: (1) cerebellum, (2) medulla oblongata, (3) hypothalamus, (4) mid brain, (5) striatum and (6) cortex.

Five of these regions will be described more precisely since they include more than one distinct anatomical structure: the 'medulla oblongata' corresponds to

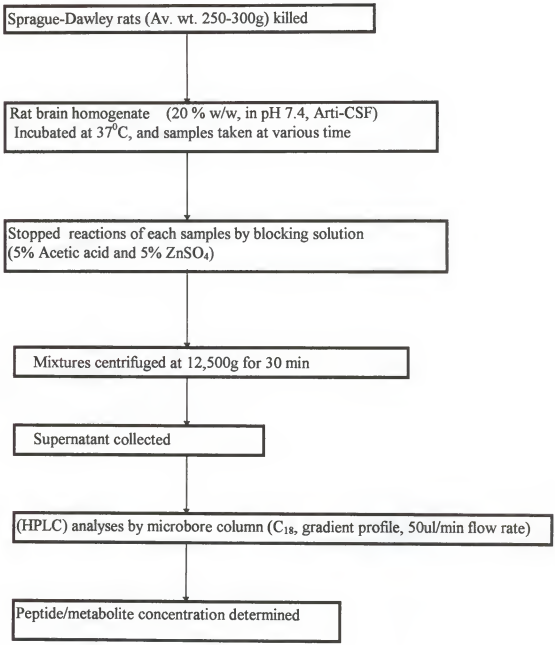

Fig 3-1 Experimental procedure in vitro

the medulla oblongata and pons; the 'midbrain' corresponds to the midbrain and thalamus and subthalamus (the latter structures being part of the diencephalon); the 'striatum' contains the putamen nucleus and caudate nucleus (striatum) and the globus pallidus nucleus (i.e., the basal ganglia of the telencephalon without the amygdala). The 'cortex' corresponds to the telecephalon without the 'striatum'; it includes white and gray matter of the cerebral cortex. The dissection of the brain was performed as follows: first the rhombencephalon (A) is separated by a transverse section from the rest of brain (Fig.3-2 Section 1) and dissected into two parts; the 'cerebellum' and the 'medulla oblongata'. A transverse section is made at the level of the 'optic chiasma' which delimits the anterior part of the hypothalamus and passes through the anterior commissure (section 2). This section separates the cerebrum into two parts, B and C. Part B is divided into five fractions. First the 'hypothalamus' is dissected by taking the anterior commissure as a horizontal reference and the line between the posterior hypothalamus and the mammillary bodies as the caudal limit (Fig.3-2). The 'striatum' is dissected with the external walls of the lateral ventricles as internal limits and the corpus callosum as external limits (Fig.3-2). The frontal parts of the striatum, which are in portion C, are dissected separately and combined with the posterior parts from portion B. The 'midbrain' is gently separated from the remaining parts of the brain. The 'hippocampus' is then dissected. The remainder of part B is combined with the remainder of part C to form the 'cortex'.

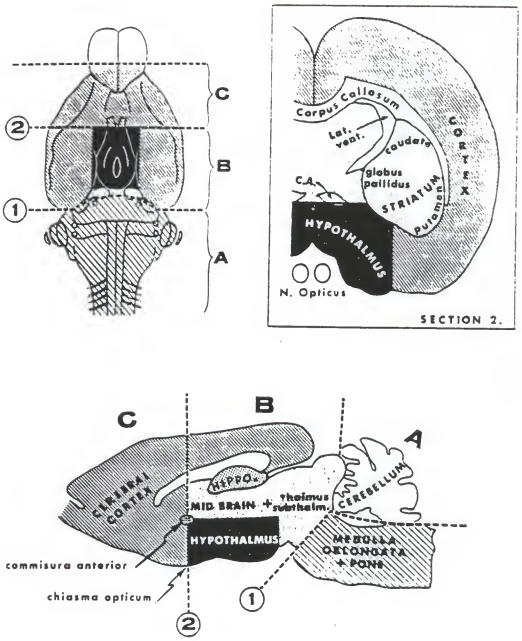

Fig 3-2 Diagrammatic representation of disection procedure for rat brain. Dotted lines indicate positions for initial section.

In the in vivo microdialysis studies, a small probe containing the dialysis membrane can easily be placed into the tissue, and a suitable perfusion fluid is continuously pumped through the probe at a low flow rate and the resulting perfusate is collected and analyzed.

Compounds to be administered can be added to the perfusion fluid and will diffuse out of the probe and into the surrounding tissue. The molecular size cutoff of the membranes excludes degradative enzymes and microorganisms from the sample, so that the chemical integrity of the compounds removed from the living tissue is preserved until sample analysis. Awake animals were used, because studying the chemistry of the tissue in a conscious, functioning animal avoided possible interference from drugs when experiments were done under anesthesia. The experimental set-up is shown schematically in Fig. 3-3.

Male Sprague-Dawley rats was used throughout the experiments. The implantation of a guide cannula was done after the animal is completely anesthetized (Ketaset, 0.1 g/kg i.p.). The top of the head is shaved and a midline cut is made through the skin. Fascia over the skull is scraped away, and the cut is protracted. With a hand-held dental drill, a hole is drilled through the skull at an appropriate location (e.g., 1.4 mm to the right of the superior sagittal sinus), and two additional hole is drilled posterior to this hole for stainless-steel anchor screws. The insertion of the microdialysis probe guide (cannula) was done by using a stereotaxic frame equipped with micromanipulator. The guide cannula was held permanently in place with cranioplastic cement filling the protracted area. The

actual process of insertion of the probe into the brain did not start until we have allowed adequate time (3-5 days) for healing after the implantation of the guide.

After inserting the microdialysis probe (CMA/12, CMA Microdialysis, Acton, MA) into the guide, the animal was placed into a containment unit (BSA, West Lafayette, IN). Microdialysis probes with a 4 mm membrane (cutoff molecular weight 20,000 Da) was used. The dialysis rate was varied between 0.25 and 5 µl/min using a syringe pump The vehicle solution was an artificial cerebrospinal fluid (146 mM Na^+, 2.7 mM K^+, 155 mM Cl^-, 1.2 mM Ca^{2+}, and 1.0 mM Mg^{2+}). Blank was collected and analyzed before each perfusion experiment. The peptide was dissolved in the artificial CSF in 5, 0.5, 0.05 mM concentration, and supplied to the brain through the microdialysis probe via polyethylene tubing (PE-50). A liquid switch was used to alternate between two syringe pumps; one delivering the perfusion fluid containing the peptide, and the other doing the perfusion with the "pure" artificial CSF to control the duration (from 5 min to 2 hours, depending of the rate of degradation) of peptide administration. Dialysates were collected manually into polypropylene centrifuge tubes under ice-cooling over 1-6 hours, and appropriate volumes was analyzed.

The analyses were performed by gradient microbore HPLC (30 cm x 1 mm i.d. C-18 column) and/or electrospray ionization (ESI) mass spectrometry. Microbore HPLC analysis were done on a system consisting of a ThermoSeparation/ SpectraPhysics (Fremont, CA) SpectraSERIES P222 binary gradient solvent delivery system, a Rheodyne (Cotai, CA) model 7125 injector

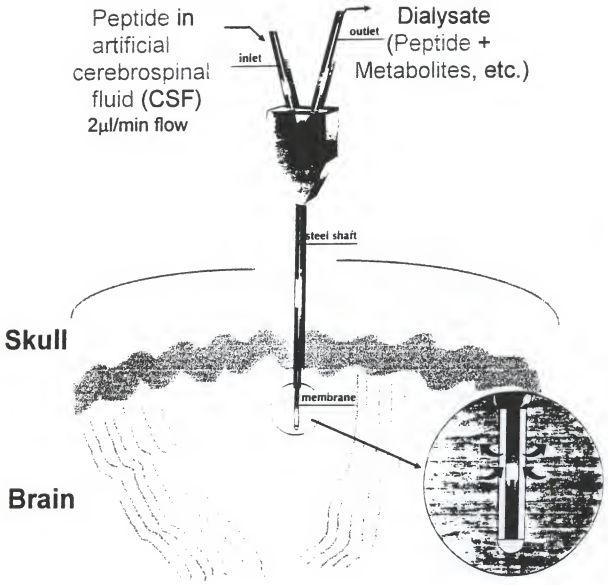

Fig 3-3 In vivo cerebral microdialysis involving delivery of peptides into the brain via the probe and simultaneous sampling of the extracellular space of the tissue

valve equipped with a 5-µl sample loop, a Spectroflow 757 variable wavelength UV/VIS detector (Kratos Analytical, Manchester, U.K.) operated at 215 nm, and a Hewlett-Packard Model HP 3395 computing integrator (Palo Alto, CA). A 30 cm x 1.0 mm i.d. Supelcosil LC-18 (5 µm) reversed-phase column (Supelco, Bellefonte, PA) was used as a flow rate of 50 µl/min that was maintained by using a dynamic split (before the injection valve) via a balance column (25 mm x 4.6 mm i.d.) connected in parallel with the microbore column. The mobile phase was mixed from 0.1 % (v/v) TFA in water and 0.08 % (v/v) TFA in acetonitrile, and gradient elution was done from 5 % to 95 % of organic modifier that changed in a linear profile at a rate of 1 %/min.

Our primary method for the identification of the metabolites in the was electrospray ionization mass spectrometry. A Vestec 200ES instrument (Vestec Corp., Houston, TX) was used to obtain the electrospray ionization (ESI) mass spectra. The sample solution was drawn into a standard laboratory syringe (100 µl, Model 1710, Hamilton Co., Reno, Nevada, U.S.A.) and supplied into the electrospray probe through a 50 cm x 0.1 mm i.d. deactivated fused silica capillary (Scientific Glass Engineering, Victoria, Australia) at 2 to 5 µl/min flow rate by a medical infusion pump (SAGE Instruments, Model 341B, Boston, MA, U.S.A.). A 0.005 in i.d. x 0.010 o.d. flat tipped hypodermic needle held at 2.4-2.8 kV potential was used to produce the electrospray spray current at ca. 10 mm distance from the sampling orifice. The source block was heated to 250^0 C, and the spray chamber temperature was regulated to 55^0 to 60^0 C by adjusting the flow of the cooling

water. A Vector/Two data system (Teknivent, St. Louis, MO, U.S.A.) was used to control the quadrupole analyzer (2000 Da mass range), and to collect mass spectra in the appropriate (usually 100-2000 Da) mass window. In order to remove inorganic contaminants adversely affecting this desorption ionization technique, solid-phase extraction (disposable Supelclean LC-18 minicolumns, supelco, Bellefonte, PA) was applied for sample purification. One-ml cartridges was used. The octadecylsilica packing was pre-wetted with 2 ml methanol, then washed with 2 ml aqueous 3 % acetic acid solution. The sample was adsorbed from the perfusate after slight acidification (acetic acid). The unretained sample constituents are removed by washing with 2 ml aqueous 3 % acetic acid solution, then the peptide(s) was eluted with methanol/aqueous 3 % acetic acid mixture. The percentage of organic solvent was adjusted so that the total elution volume of sample is 300-400 µl. The sample recovery of the solid-phase extraction procedure is high, usually exceeds 80 percent. If the sample becomes highly diluted, the solvent is removed by a stream of nitrogen, the residue is taken up with small amount (ca. 50 µl) methanol/aqueous 3 % acetic acid mixture, and analyzed by ESI-mass spectrometry.

The ESI mass spectra of peptide mixtures obtained from microdialysis experiments may be too complex to interpret (because of the multiple charging of peptides) without using analytical separation, such as HPLC, before the mass spectrometric analysis. However, ESI mass spectrometry only permits the introduction of a liquid flow in the 1 to 10 µl/min flow rate. In terms of the HPLC technique, this is the working range of packed capillary columns with diameters

(i.d.) of 180-320 microns (μLC). The perfusates was injected via a Rheodyne 7125 valve (5-, 20- or 50-μl sample loop) onto a 2 cm x 320 μm i.d. octadecylsilica (C_{18}) μ-Precolumn (LC Packings, San Francisco, CA) directly connected to the injector. Acidified water (0.05 % formic acid, v/w) was used to preconcentrate the sample on the μ-precolumn. The flow rate of 10 μl/min was delivered by a conventional gradient pump (Spectroflow 400) equipped with a low-pressure gradient former (Spectroflow 430, Kratos Analytical, Manchester, U.K.), and an ACURATE^{TM} microflow processor (LC Packings, San Francisco, CA). During the preconcentration (10-20 min), the effluent was discarded. The flow was then stopped, and a 15 cm x 320 μm (or 180 μm) i.d. analytical C_{18} column was connected to the precolumn with a high-pressure, zero dead-volume connector. The flow rate was set to 5 μl/min (2 μl/min with the 180 μm i.d. column), and the gradient program (0 to 50-70 % acetonitrile, 1-2 %/min rate) was started. The outlet capillary (75 μm i.d. x 280 μm o.d. x ca. 30 cm long fused silica) was connected to the electrospray probe. Successive ESI mass spectra was recorded and stored by the Vector\Two data system for the identification of the separated metabolites.

In addition, solid-phase peptide synthesis was used to obtain substrates for the stability studies (Prokai-Tatrai et al., 1996). A Synthor 2000 (Peptide International, Louisville, KY) instrument was used. The synthesis used the Fmoc chemistry on Wang resin (COOH terminal). After cleaving the product from the resin with 95% TFA + 5% water, preparative HPLC purification were performed

on a system consisting of a ThermoSeparation/Spectra-Physics (Fermont, CA) SpectraSERIES P200 binary gradient pump, a Rheodyne (Cotati, CA) Model 7125 Injector equipped with a 5-ml loop, and a Spectra Physics Spectra 100 UV/VIS detector operated with a preparative flow cell at 216 nm. The preparative column was packed a 15-μm Delta-Pak C_{18} (25 mm x 100 mm cartridge with a Guard-Pak insert from Waters Chromatography). The mobile phase was mixed from 0.1% (v/v) of TFA in H_2O (A) and 0.08% (v/v) of TFA in CH_3CN (B) to give a linear gradient from 95% A/5% B to 5%A/95% B by increasing the organic modifier at 2%/min rate. The flow rate was 5.0 ml/min. The solutions of the collected products were lyophilized and characterized by ESI mass spectrometry and analytical (microbore) gradient HPLC.

The ESI mass spectrometric analysis was also complmented with matrix-assisted laser desorption ionization (MALDI) mass spectrometry for large (Mr > 1,000 Da) peptides. A PerSeptive Biosystems (Framingham, MA) time-of-flight instrument Vayager, with delayed extraction and reflector) was used. The sample (1 μl) was mixed with a matrix solution (10 μl) for analysis. The matrix (α-cyano-4-hydroxyannamic acid) solution was prepared in 5 g/L concentration dissolved in 33% acetonitrile/ 67% water con taining 0.1% TFA. A droplet (~2 μl) of the sample/matrix mixture was transferred on the stainless steel sample plate and allowed to dry. Pulsed laser irradiation (355 nm wavelength) was applied, and successive acquisition of MALDI mass spectra was done. At least ten spectra were averaged for evaluation.

In studies on the metabolism of synthetic dynorphin A peptides in brain tissue in vitro and in vivo, Dyn A (1-13), (1-10) and (1-6) were obtained from Sigma (St. Louis, MO), Dyn A (1-12) and (1-11) were purchased from Peninsula Laboratories (Blemont, CA), and Dyn A (2-13) was acquired from Bachem Bioscience (King of Prussia, PA). Each peptide was of >98% purity based on gradient RPLC. Water was purified by ion exchange and filtering through active-carbon packed cartridges. Acetonitrile was of HPLC grade, all other chemicals were of analytical reagent grade and were obtained from Fisher Scientific (Pittsburgh, PA).

CHAPTER 4

RESULTS AND DISCUSSION

Evaluation of the In Vitro Approach

Studies of peptide degradation in brain homogenate were straightforward to carry out, once the substrate was added to the medium in concentration optimal for the analytical technique chosen.

For microbore HPLC (with UV detection), most studies can be done with peptide concentration from 10 to 100 μM in brain homogenate. However, the protein precipitation after sampling (blocking solution) needed to be optimized. Otherwise, self-proteolysis of the brain tissue during incubation was a serious source of contamination that made the HPLC chromatograms difficult to evaluate for the presence of peptide metabolites. The natural opioid peptide, Leu-enkephalin, yielded the expected major metabolite, Gly-Gly-Phe-Leu-OH, whose increase of concentrations were easy to measure together with the decrease of the concentration of the parent peptide (Fig. 4-1 (a)). However, other metabolites were impossible to detect.

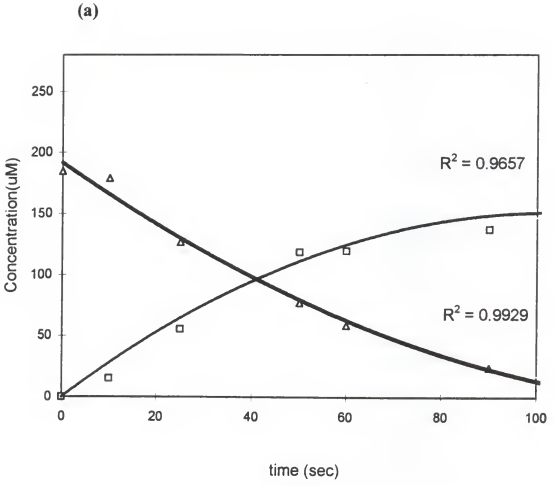

Fig 4-1 (a) In vitro degradation of Leu-enkephalin and its metabolically stable analogs: Tyr-Gly-Gly-Phe-Leu-OH (Δ) and Gly-Gly-Phe-Leu-OH (□) as a metabolite (20% rat brain homogenate, pH 7.4, 37°C), (b) In vitro degradation of Leu-enkephalin and its metabolically stable analogs: Tyr-D-Ala-Gly-Phe-Leu-OH (20% rat brain homogenate, pH 7.4, 37°C) and (c) In vitro degradation of Leu-enkephalin and its metabolically stable analogs: Tyr-D-Ala-Gly-Phe-D-Leu-OH (20% rat brain homogenate, pH 7.4, 37°C)

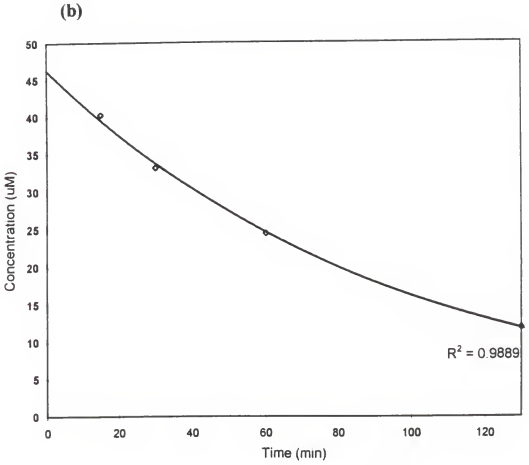

Fig 4-1 --continued

(c)

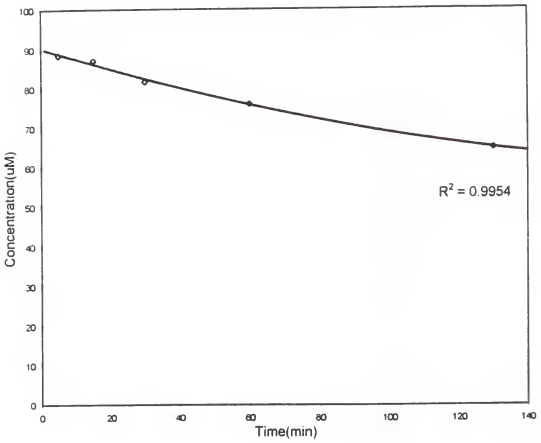

Fig 4-1 --continued

Metabolism of five synthetic dynorphins have been studied in 10% brain homogenate in vitro. Kinetic data on their degradation in brain homogenate were given in Table 4-1. Identification of the major metabolites by collecting fractions after gradient microbore RPLC only revealed certain major, relatively long-lived metabolites, as shown in Fig. 4-2. The smaller or rapidly appearing then disappearing metabolites could not be isolated in amounts necessary for positive identification by their ESI mass spectra. The tissue also gave numerous peaks that interfered with the HPLC analysis. Even after on-line LC/ESI-MS analysis, identification of many metabolites were possible via systematic screening for their presence (i.e., displaying reconstructed ion chromatograms of the expected metabolites), and several samples had to be analyzed for complete metabolic profiling.

The in vitro approach has been satisfactory for studying the degradation kinetics of synthetic dynorphins. Using brain homogenate, self-proteolysis of the brain tissue during incubation was a serious source of contamination, which made the HPLC chromatograms and ESI mass spectra difficult to evaluate for the presence of several key (especially short-lived) peptide metabolites. The value of this approach is that biological stability of dynorphins can be compared (Table 4-1), although homogenates may also contain many cytosolic peptidases to which, in vivo, dynorphins may not be exposed.

Table 4-1. Stability of selected synthetic dynorphins in rat brain homogenate at pH 7.4 and 37°C (Determined by microbore RPLC assay, conditions as in Fig. 4-6).

Peptide	Rate Constant (min^{-1})	Half-life (min ± standard error, N=3)
Dyn A (1-13)	0.24	2.9 ± 0.7
Dyn A (1-12)	0.13	5.6 ± 0.5
Dyn A (1-11)	0.47	1.5 ± 0.5
Dyn A (1-10)	0.48	1.5 ± 0.5
Dyn A (2-13)	0.23	3.0 ± 0.8

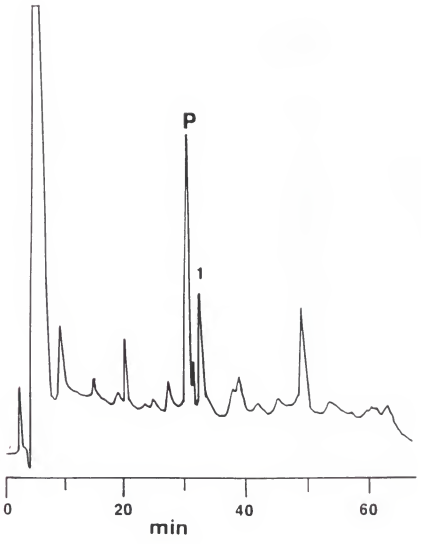

Fig. 4-2 Microbore HPLC analysis of the degradation products from incubation of Dyn A (1-13) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu, YGGFLRRIRPKLK) in rat brain homogenate (20%, w/w, pH 7.4, 37/C). HPLC conditions: 30 cm x 1.0 mm I.D. Supelcosil LC-18 column, 50 µl/min flow rate, gradient elution from 5 to 95% acetonitrile (0.1 to 0.08 % TFA) at 1%/min, UV detection at 216 nm. P: parent peptide (Dyn A 1-13), 1: Identified as Dyn A (1-12) based on ESI mass spectrum (*m/z* 740.4 and 492.9; M_r 1477.3).

For metabolically stable analogs, including [D-Ala^2]-Leu-enkephalin (DALLE), [D-Ala^2] [D-Leu^5]-enkephalin (DADLE), sandostatin, Lan-sandostatin and DPDPE, quantitative analysis of the metabolites obtained was not possible because of the slow degradation of the parent compounds (Fig 4-1 b-c). The metabolites were buried in the chromatographic background coming from the brain homogenate.

In summary, the in vitro approach was suitable for measuring the stability and obtaining kinetic data (k, $t_{1/2}$). However, detection of a full set of metabolites formed during degradation could not be done for most of the peptides studied.

Evaluation of the *In Vivo* Technique

This new approach for studying peptide metabolism in the brain (Andren and Caprioli, 1995) has not been thoroughly evaluated. No information is available on how microdialysis conditions should be set for delivering the required amount of peptide to the tissue and recovering the metabolites. The two principal parameters that can be adjusted for the experiments are the concentration of peptide in the perfusion fluid and the perfusion flow rate. The concentrations of Leu-enkephalin and its major metabolite (GGFL) in the perfusates as a function of peptide concentration and flow rate are given in the charts of Fig 4-3. The system is most predictable when relatively large peptide concentrations (1-5 mM) in the

perfusion fluids are applied (Fig 4.3 (a)). Although more than 50% of the peptide in the perfusion fluid can be delivered to the tissue at low (0.25-0.5 μl/min) flow rates, there may be a substantial drop in the concentration of the recovered metabolite in the microdialysate. In addition, sufficient volume of sample is collected in relatively long time, as compared to higher flow rates. The flow rate of 0.8 μl/min appears to be the optimal for the experiments.

The microdialysates collected under these conditions show no interference from a chromatographic background, as shown in Fig. 4-4.

An HPLC/ESI-MS analysis also revealed the identity of products from enzymatic degradations to which the peptide is less susceptible. The mass chromatograms shown in Fig 4-5 indicate that carboxypeptidase cleavage that gives Tyr-Gly-Gly-Phe-OH (m/z 443) is about 200-fold less abundant than the aminopeptidase cleavage (m/z 393). Even a secondary metabolite (Gly-Gly-Phe, m/z 280 for the protonated molecule) can be identified (Fig 4-6). The practically background-free microdialysates make the identification of the peptides straightforward. However, these studies cannot replace in vitro investigations, because quantitative stability data (k and $t_{1/2}$) cannot be obtained by microdialysis.

ESI mass spectra obtained from the microdialysates revealed the presence of numerous brain metabolites, as shown in Fig. 4-2 (these products were not present in the corresponding control samples). Several major metabolites of Dyn A (1-13) and Dyn A (1-11) could be identified based on their molecular ions ($[M+H]^+$, $[M+2H]^{2+}$ and/or $[M+3H]^{3+}$) from the mass spectra of the peptide

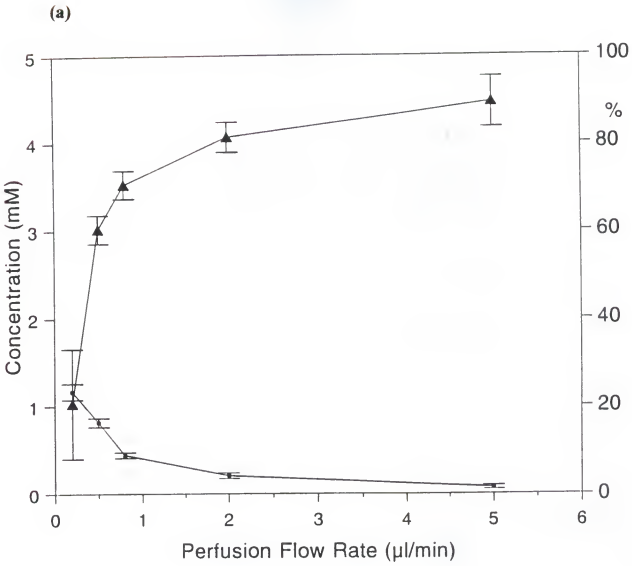

Fig 4-3 Effect of Leu-enkephalin concentration in perfusion and flow rate on delivery to the tissue (♦) and recovery of the major metabolite (▲). Initial peptide concentration: (a)-5mM, (b)-500µM, (c)-50µM

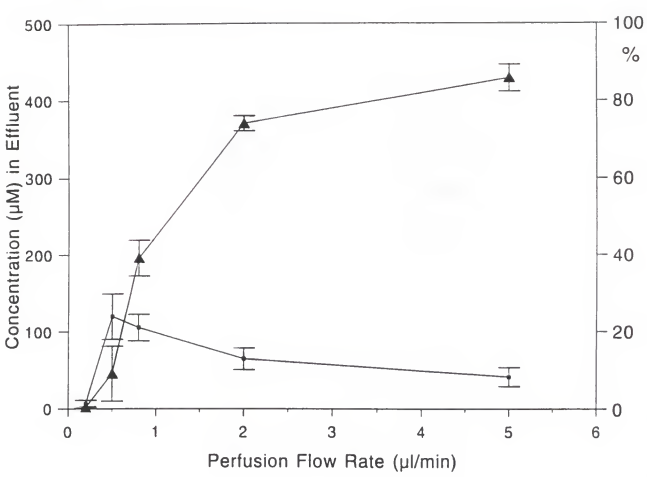

Fig 4-3 --continued

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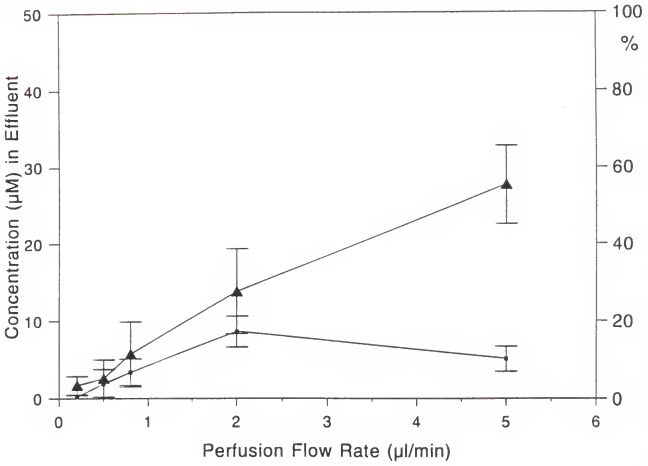

Fig 4-3 --continued

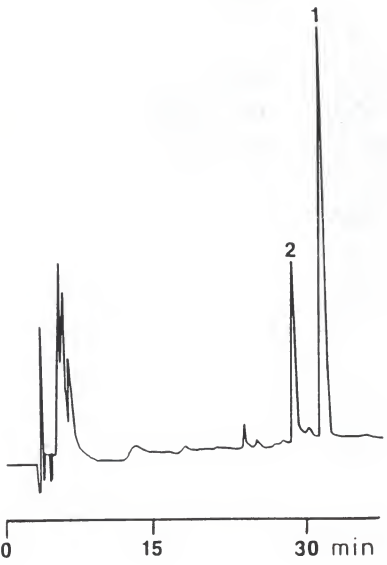

Fig. 4-4 HPLC chromatogram of the microdialysate after perfusion of the probe with Leu-enkephalin (500μM, 0.8μl/min). 1; Leu-enkephalin, 2; Des-Tyr-enkephalin (GGFL)

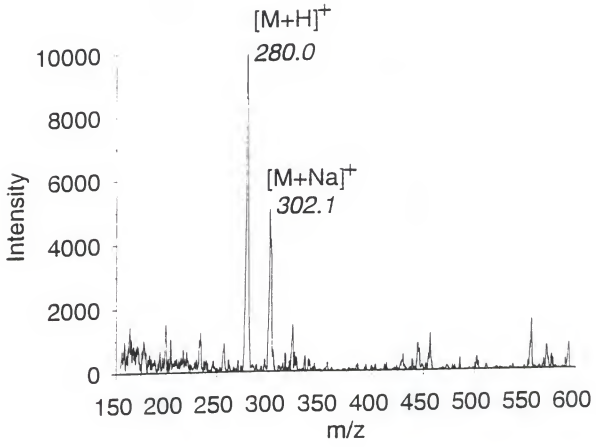

Fig. 4-5 HPLC/ESI-MS of the microdialysate after Leu-enkephalin perfusion (5mM, 0.8μl/min) into the globus pallidus.

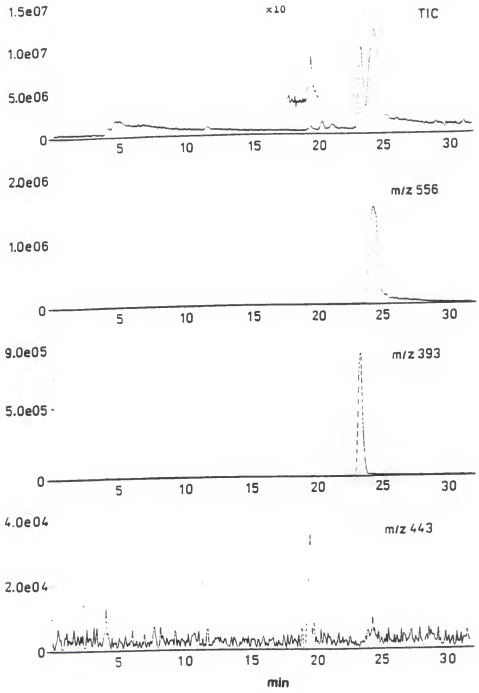

Fig. 4-6 ESI mass spectrum of the HPLC peak at 21 min in the chromatogram in Fig. 4-5

mixtures obtained. Shallow-gradient (0.5%/min increase in the organic modifier of the mobile phase) microbore RPLC analyses confirmed the presence of these metabolites which also coeluted with the corresponding synthetic peptides or the degradation products separated and identified from the in vitro experiments.

However, the metabolic fate of Dyn A 1-12 were difficult to evaluate because of the extremely complex ESI mass spectrum obtained after the direct analysis (i.e., without chromatographic separation) of the microdialysate (Fig. 4-7b), because of formation of ions with multiple charges and the large number of primary and secondary metabolites formed. RPLC/ESI-MS analysis facilitated the identification of metabolites. The number of peptides whose elution overlapped during the acquisition of the successive scans usually did not exceed three; thus deconvolution of the mass spectra to obtain relative molecular masses of the cleavage product was straightforward. After compiling a list of the metabolites identified, ion (mass) chromatograms were reconstructed for confirmation. Fig 4-7 shows the reconstructed ion chromatograms (RIC) for the metabolites obtained from the parent peptide (Dyn A 1-12) through the cleavage of a single peptide bond (primary products). Although the RIC peak areas may indicate the relative rates of metabolite formation by the individual peptidases involved, one must be aware of the differing dialysis efficiencies as the function of peptide size, and that ionization efficiencies under electrospray conditions also vary among peptides. On the other hand, mass-specific detection after RPLC was essential, because

complete RPLC separation of the metabolites was not achieved even by using a shallow gradient (+0.5%/min in acetonitrile).

In vivo cerebral microdialysis circumvents the problems associated with the in vitro approach. ESI mass spectrometry (after desalting) have revealed the major extracellular metabolites directly for certain synthetic dynorphins such as Dyn A 1-13 and Dyn A 1-11, as confirmed by a follow-up microbore RPLC study. However, metabolic profiling by LC/ESI-MS has been necessary to fully characterize complex mixtures, such as the one obtained during the study on striatal metabolism of Dyn A (1-12) where nine primary metabolites have been identified. The rich spectrum of the extracellular metabolism of this peptide in the brain may be connected to its relatively higher stability in the tissue, compared to related synthetic dynorphins (Table 4-1), facilitating the attack by a variety of peptidases.

The MALDI mass spectrum of Dyn A (1-13), Dyn A (1-12) and Dyn A (1-8) are also shown in Figure 4-9, 10, and 11. Because they give singly-charged molecular ions ($[M+H]^+$), the MALDI mass spectra are easier to interpret than ESI-mass spectra (Table 4-3, 4, 5).

In biodegradation studies of sandostatin and lanthionine-sandostatin, the main degradation product of sandostatin in vivo corresponds to the heptapeptide formed by enzymatic cleavage of the N-terminal D-Phe [protonated molecule, MH^+ found at m/z 872.1 in the ESI mass spectrum, expected m/z 872.1]. The lanthionine-sandostatin also showed the loss of the N-terminal D-Phe (MH+ found

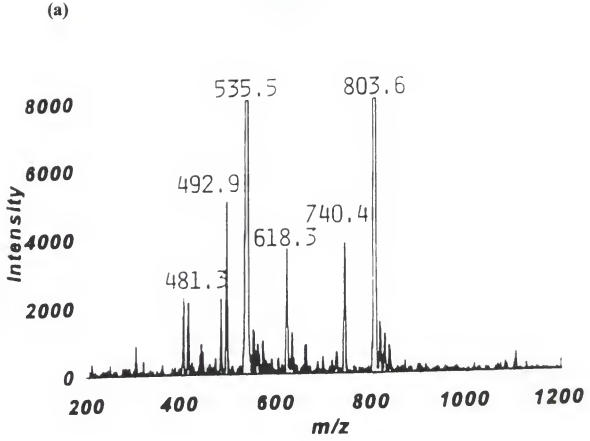

Fig. 4-7 ESI mass spectrometric analysis of the microdialysates collected by perfusion of the probes with (a) Dyn A (1-13), (b) Dyn A (1-12), and (c) Dyn A (1-8). Perfusion conditions: 1 nmol/µl peptide in artificial CSF, 0.8 µl/min flow rate, sample collected 30 min after starting peptide; duration: 60 min. The dialysates were desalted on a reversed-phase cartridge (1-µl Supelclean LC-18) before analysis.

(b)

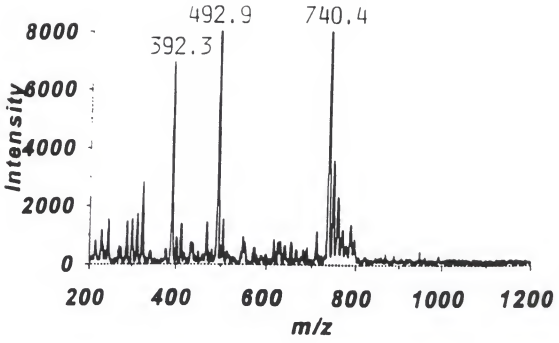

Fig. 4-7 -- continued

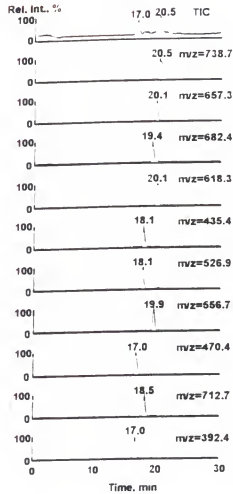

Fig. 4-8 .Total ion current (TIC, a) and reconstructed ion chromatograms (b - g) from the gradient reversed-phase HPLC/ESI-MS analysis of the microdialysates collected from rat striatum after perfusion of the probe with 100 pmol/μl Dyn A 1-12 (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu, YGGFLRRIRPKL) at 0.8 μl/min. HPLC conditions: 15 cm x 0.3 mm I.D. C-18 reversed-phase column, 6.3 μl/min flow rate, gradient elution from 5 to 95% acetonitrile (0.1 to 0.05 % TFA) at 2%/min. Reconstructed ion chromatograms: (b) *Parent peptide:* Dyn A 1-12 (m/z 738.7, doubly-charged); *Metabolites:* (c) Dyn A 1-10 (m/z 618.3, doubly-charged); (d) Dyn A 1-7 (m/z 435.4, doubly-charged); (e) Dyn A 5-12 (m/z 526.9, singly-charged); (f) Dyn A 1-5 (m/z 556.7, singly-charged); (g) Dyn A 6-12 (m/z 470.4, doubly-charged); (h) Dyn A 1-6 (m/z 712.7, singly-charged); (i) Dyn A 7-12 (m/z 392.4, doubly-charged); (j) Dyn A 2-12 (m/z 657.3, doubly-charged); (k) Dyn A 1-11 (m/z 682.3, doubly-charged).

Table 4-2. Major ESI Ions of Synthetic Dynorphins

Peptide	Sequence	ESI Ions
Dyn A 1-13	YGGFLRRIRPKLK	803.6 (2+), 535.5 (3+), 401
Dyn A 1-12	YGGFLRRIRPKL	740.4 (2+), 492.9 (3+)
Dyn A 2-13	GGFLRRIRPKLK	722.9 (2+), 481.3 (3+)
Dyn A 1-10	YGGFLRRIRP	618.3 (2+), 412.5 (3+)
Dyn A 1-11	YGGFLRRIRPK	682.0 (2+), 455.1(3+)
Dyn A 2-11	GGFLRRIRPK	600.4 (2+), 400.3 (3+)
Dyn A 1-6	YGGFLR	712.0 (1+)
Dyn A 6-12	RIRPKL	392.3 (2+)

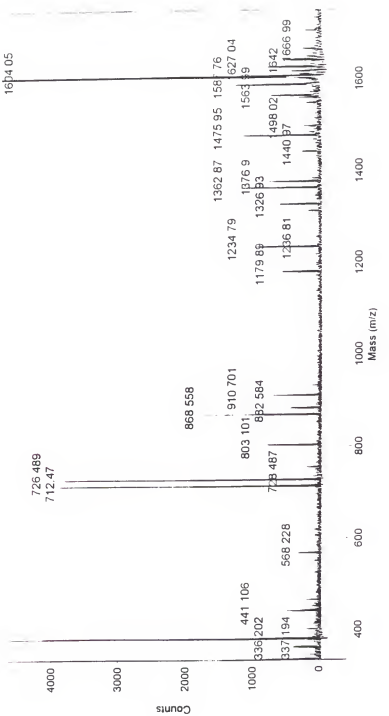

Fig 4-9. MALDI mass spectrum of Dyn A (1-13) showing the release of the its metabolites after in vivo cerebral microdialysis (rat striatum).

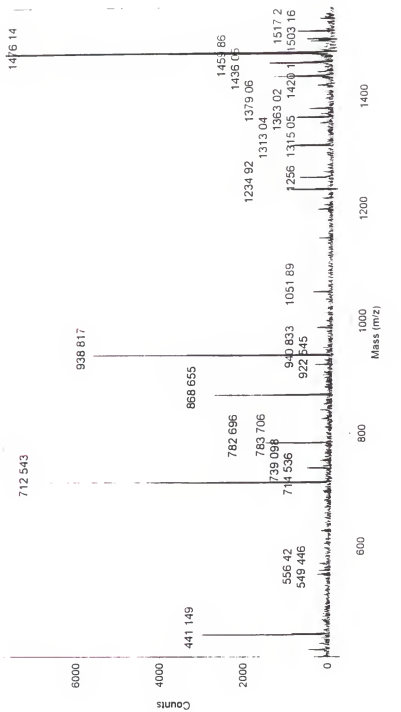

Fig 4-10. MALDI mass spectrum of Dyn A (1-12) showing the release of the its metabolites after in vivo cerebral microdialysis (rat striatum).

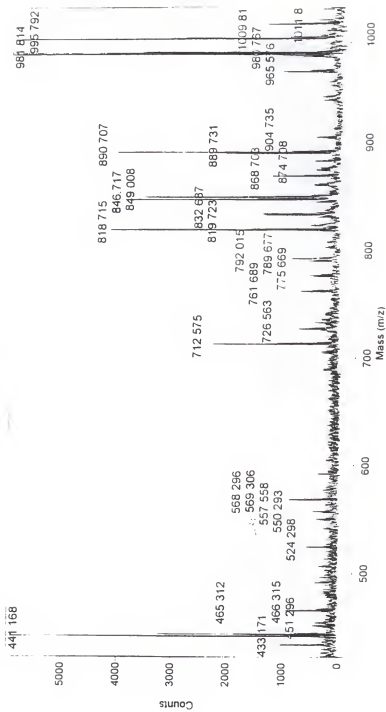

Fig 4-11. MALDI mass spectrum of Dyn A (1-8) showing the release of the its metabolites after in vivo cerebral microdialysis (rat striatum).

Table 4-3. Major MALDI Ions of Synthetic Dynorphins from Fig 4-9.

Peptide	Sequence	$[M+H]^+$ (m/z)
Dyn A 1-13	YGGFLRRIRPKLK	1604.1
Dyn A 1-12	YGGFLRRIRPKL	1476.1
Dyn A 2-13	GGFLRRIRPKLK	1440.1
Dyn A 1-10	YGGFLRRIRP	1180.0
Dyn A 1-11	YGGFLRRIRPK	1327.0
Dyn A 1-6	YGGFLR	712.0
Dyn A 5-12	RRIRPKL	868.7
Dyn A 6-12	RIRPKL	782.6

(c)

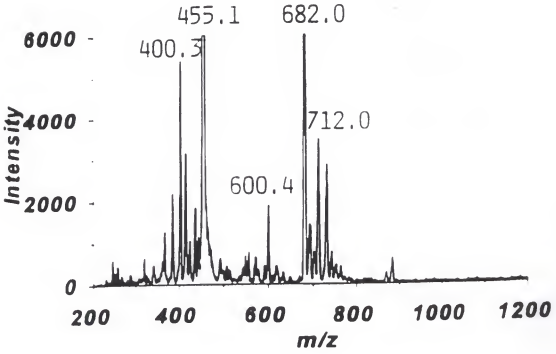

Fig. 4-7 --continued

Table 4-4. Major MALDI Ions of Synthetic Dynorphins from Fig 4-10.

Peptide	Sequence	$[M+H]^+$ (m/z)
Dyn A 1-12	YGGFLRRIRPKL	1476.1
Dyn A 2-12	GGFLRRIRPKL	1313.0
Dyn A 1-10	YGGFLRRIRP	1180.0
Dyn A 1-11	YGGFLRRIRPK	1327.0
Dyn A 1-6	YGGFLR	712.0
Dyn A 1-5	YGGFL	556.4
Dyn A 7-12	RIRPKL	782.6
Dyn A 6-12	RRIRPKL	868.7

Table 4-5. Major MALDI Ions of Synthetic Dynorphins from Fig 4-11

Peptide	Sequence	$[M+H]^+$ (m/z)
Dyn A 1-8	YGGFLRRI	981.8
Dyn A 2-8	GGFLRRI	818.7
Dyn A 1-7	YGGFLRR	868.7
Dyn A 1-6	YGGFLR	712.0
Dyn A 1-5	YGGFL	557.5

at m/z 839.6 expected m/z 840) as a major metabolite. No other metabolites by cleavage of peptide bonds inside of the disulfide-bridged and the lanthionine cyclopeptide were detected.

The metabolic stability of sandostatin was expected to be increased by introducing a lanthionine bridge in the place of the disulfide moiety. Our previous studies on lanthionine opioids indicated that the lanthionine molecules are significantly more stable toward enzymatic degradationthan their disulfide-bridged analogs. Bauer et al. reported in 1982 that sandostatin is many times more stable than the natural somatostatin-14 in an ultrafiltrate of rat kidney homogenate. In our study, the in vitro stability of lanthionine-sandostatin was compared with sandostatin against degradation by rat brain homogenate. Both compounds have clearly a prolonged duration of action. However, the lanthionine analog showed a remarkable 2.4 times longer half life than sandostatin. In vivo metabolism of sandostatin in rat brain tissue was studied by utilizing the cerebral microdialysis method. Both peptides yield two extracellular metabolites detected by ESI-MS from the collected dialysates (Fig. 4-12). The exocyclic residues (D-Phe and Thr-ol) show enzymatic instability; they are cleaved enzymatically from the parent compounds and provide the corresponding hexa- and heptapeptides as major metabolites. Only small protions of both drugs degraded in vivo in rat brain, which highlights the increased stability of these molecules.

In conclusion, the in vitro and in vivo techniques should be regarded as complementary. The simultaneous approach has been useful in characterizing the

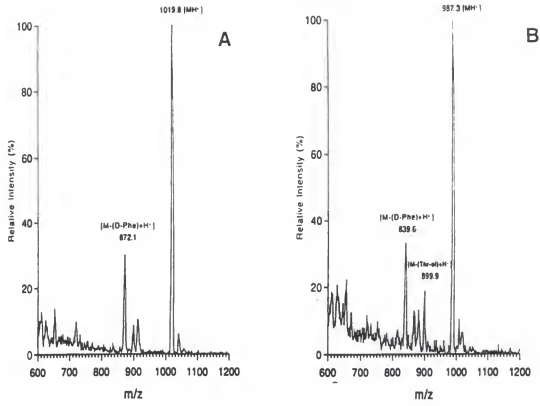

Fig 4-12 Molecular ion (MH^+) regions of the ESI mass spectra form dialysates colleted during infusion of sandostatin (A) and lanthionine-sandostatin (B) in to rat brain (striatum).

brain-metabolism of various peptides and peptide analogs during the validation of the methods.

Metabolic Optimization of Brain-Targeting

Brain-Targeting of Leucine-Enkephalin Analogs : Probing Conjugate Stability and Peptide Release in the Brain

The stability data of the peptide conjugates responsible for the release of the enkephalin analog in the brain are collected in Tables 4-6,7, together with the major degradation products identified by ESI mass spectrometry. In vitro studies only allowed us to find out whether the biologically active peptide was among the products released from the trigonellyl conjugate, when the stability of the conjugate exceeded 4 hours, as in case of T^+-A-YaGFL shown in Fig. 4-13. No other metabolites could be found without specific "screening". This was due to the presence of a "background" from the brain homogenate, which was getting more and more interfering with the increase of the time of incubation. However, in vivo microdialysis combined with ESI mass spectrometry was not only suitable to find out whether the peptide was released from the trigonellyl compound (even in the case of T^+-A-YaGFL, where the release of DALLE was not detected in vitro), but also to find some other metabolites. the enkephalin analog in the brain are collected in Table 4-8, together with the major degradation products identified by ESI mass spectrometry.

Table 4-6 Peptide and peptide analogs for in vitro CNS degradation

Peptide	Stability in brain-homogenate		Major metabolites
	$t_{1/2}$ (min)	k	
Leu-enkephalin (YGGFL)	0.5	1.386	GGFL, (YGGF)
Met-enkephalin (YGGFM)	0.5	1.386	GGFM, (YGGF)
Des-Tyr-enkephalin (GGFL)	1.4	0.495	GGF
DALLE (YdAGFL)	76	0.009	N.D.
DADLE (YdAGFdL)	726	0.00095	N.D.
[D-PEN^2, D-PEN^5]enkephalin	1192	0.00058	N.D
Sandostatin [1]	1711	0.0004	N.D.
Lanthionine-Sandostatin [1]	1465	0.00047	N.D.
Dyn 1-13 (YGGFLRRIRPKLK)	2.9	0.24	Dyn 1-12
Dyn 1-12 (YGGFLRRIRPKL)	5.6	0.123	Dyn 1-10
Dyn 1-11 (YGGFLRRIRPK)	1.4	0.495	Dyn 2-11
Dyn 1-10 (YGGFLRRIRP)	1.3	0.533	Dyn 2-10
Dyn 1-8 (YGGFLRRI)	0.7	0.99	Dyn 2-8, YGGFL
Dyn 2-13 (GGFLRRIRPKLK)	3.0	0.23	N.D.

*[1] was published in J. Med. Chem, Osapay et al., 1997

Table 4-7 Peptide and peptide analogs for in vivo microdialysis approach

Peptide	Major metabolites
Leu-enkephalin (YGGFL)	GGFL, (YGGF)
Met-enkephalin (YGGFM)	GGFM, (YGGF)
Des-Tyr- enkephalin (GGFL)	GGF
DALLE (YdAGFL)	dAGFL
DADLE (YdAGFdL)	dAGFdL
[D-PEN^2, D-PEN^5]enkephalin	N. D.
Sandostatin [1]	Des-dF-Sandostatin
Lanthionine-Sandostatin [1]	Des-dF-Lan-Sandostatin
Dyn 1-13 (YGGFLRRIRPKLK)	Dyn 1-12
Dyn 1-12 (YGGFLRRIRPKL)	Dyn 1-10
Dyn 1-11 (YGGFLRRIRPK)	Dyn 2-11
Dyn 1-10 (YGGFLRRIRP)	Dyn 2-10
Dyn 1-8 (YGGFLRRI)	Dyn 2-8, YGGFL
Dyn 2-13 (GGFLRRIRPKLK)	

*[1] was published in J. Med. Chem, Osapay, Prokai, Kim et al., 1997

In vitro studies only allowed us to determine whether the biologically active peptide was among the products released from the trigonellyl conjugate, when the stability of the conjugate exceeded 4 hours, as in case of T^{+}-A-YaGFL shown in Fig. 4-13. No other metabolites could be found without specific "screening". This was due to the presence of a "background" from the brain homogenate, which was getting more and more interfering with the increase of the time of incubation. However, in vivo microdialysis combined with ESI mass spectrometry was not only suitable to find out whether the peptide was released from the trigonellyl compound (even in the case of T^{+}-A-YaGFL, where the release of DALLE was not detected in vitro), but also to find some other metabolites.

As shown in Fig. 4-14, the removal of the trigonellyl from the conjugate is clearly one of the possible reactions. This may represent an amidase cleavage. However, amidase activity in the brain is low, and it is presumed that the enzyme responsible for the removal of the trigonellyl from the peptide chain is an aminopeptidase. Aminopeptidases are abundant in the brain, and this finding (Fig. 4-14) corroborates the hypothesis in the CDS design that the N-terminal trigonellyl may be considered a substitute for an amino acid residue. The tetrapeptide D-Ala-Gly-Phe-D-Leu-OH is from the aminopeptidase cleavage of DADLE, based on the in vivo metabolism study completed as part of the method development (Chapter 3).

HPLC profiles of T^+-[Pro-Pro^0]-[D-Ala^2]-[D-Leu^5]-enkephalin showed the appearance of [D-Ala^2]-[D-Leu^5]-enkephalin as the major metabolite. However, interference from proteins/peptides in brain homogenate hampered the identification of other possible metabolites. Therefore, to find a method to prevent this high level of contamination, microdialysis has been used. The *in vitro* and *in vivo* experiment showed agreement in terms of the major metabolite identified by HPLC retention times

For conjugates with spacers intended to be cleaved by prolyl endopeptidase (PP, PA, AP and AA), half-lives were one to two orders of magnitude shorter than that of the dipeptidyl peptidase degradation (Table 4-8). In the meantime, the simultaneous release of DADLE was obtained, as shown in Fig. 4-15.

In distribution of prolyl endopeptidase activity within the brain, metabolites was checked by the methods discribed in Chapter 3. Each different area (as 20% homogenate) was incubated with 100 μM of T^+-Pro-Pro-DADLE, and periodical sampling was done. The sample was centrifuged and supernatant was analysed. The results as shown in Table 4-10. The conjugate has lowest half life ($t_{1/2}$=4.3 min) in the cortex area and medulla oblongata area shows the highest half life ($t_{1/2}$=72 min). The cerebellum area shows about two-fold reduction in enzyme activity compared to the cortex area.

Table 4-8 Stability and major metabolite(s) in brain homogenate of T^+-(S)-Peptide conjugates

Peptide	Stability in brain-homogenate		Major metabolites
	$t_{1/2}$ (min)	k	
T^+-A-YaGFL	30	0.0231	T^+-A-YaG, T^+-A-YaGF-NH_2
T^+-A-YaGFl	963	0.0007	YaGFl, A-YaGFl
T^+-P-YaGFl	293	0.002	N.D.
T^+-HyP-YaGFl	3915	0.00018	N.D.
T^+-PP-YaGFl	9	0.077	YaGFl
T^+-AA-YaGFl	23	0.03	YaGFl
T^+-PA-YaGFl	649	0.001	N.D
T^+-AP-YaGFl	13	0.053	YaGFl
T^+-PHyP-YaGFl	481	0.014	N.D
T^+ -YaGFl	4707	0.03	N.D

A; Ala, a; D-Ala, F; Phe, G; Gly, L; Leu, l; D-Leu, P; Pro, Y; Tyr, T^+; trigonellyl, N.D.; not yet determined.

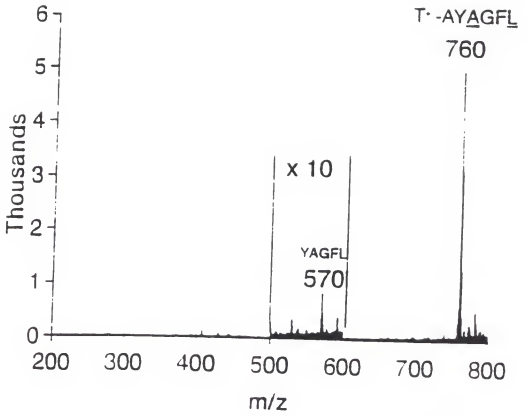

Fig 4-13 ESI mass spectrum showing the release of the enkephalin analog from the peptide conjugate (m/z 760) in vitro (rat brain homogenate, 37°C, 1 hour incubation)

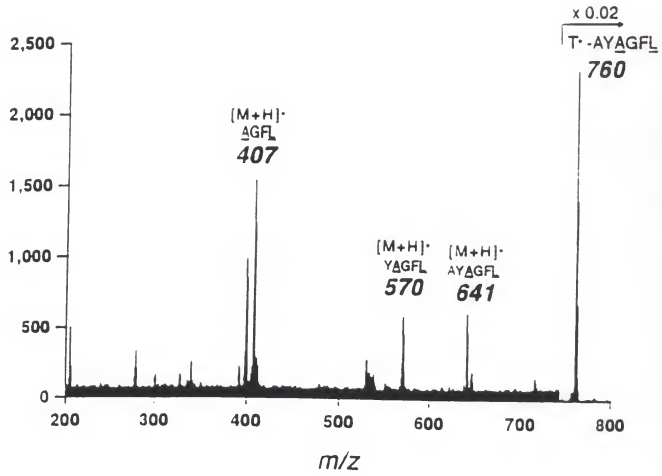

Fig. 4-14 ESI mass spectrum of T⁺-Ala-Tyr-[D-Ala]-Gly-Phe-[D-Leu] solution (5 mM) after in vivo cerebral microdialysis (rat striatum).

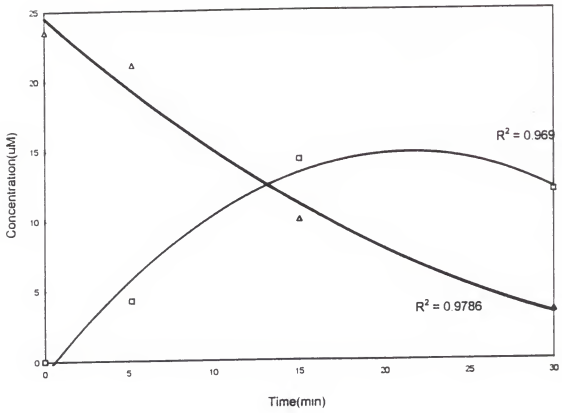

Fig. 4-15 In vitro degradation of the T*-Pro-Pro-Tyr-[D-Ala]-Gly-Phe-[D-Leu]-OH ($t_{1/2}$=9min) (Δ) and concomitant formation of Tyr-[D-Ala]-Gly-Phe-[D-Leu]-OH (□) as metabolite (rat brain homogenate, 37^{0}C, 1 hour incubation)

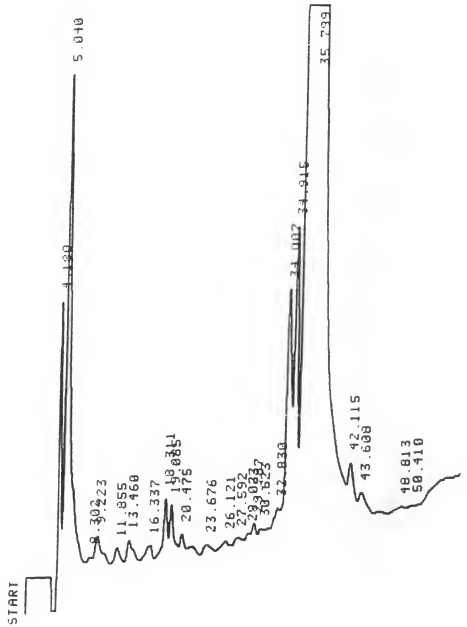

Fig 4-16 Microdyalysis experiments with T*-Pro-Pro-DADLE in striatum (HPLC chromatogram)

However, the measured first-order rate-constant (k) in cerebellum was still higher than that in whole brain. Striatum and midbrain showed similar rate of reaction, a slightly slower degradation than the one observed in cerebellum, and hypothalamus showed about 4 times slower degradation compared to cerebellum. The rate of reaction in medulla oblongata area were about 18 times slower than that in cortex area.

These results indicate the unequal regional distribution of PPCE (post-prolyl cleaving enzyme) activity against T^{+}-Pro-Pro-DADLE which results in the formation of DADLE, an opioid peptide analog.

An example of the reproducibility of the dissection procedure is given in Table 4-9. The variations in weight for the different regions for a series of three dissections ranged from 8 to 13 per cent.

In conclusion, the results obtained in this study confirm and extend the previous findings in the whole brain, and indicate that there are considerable variations in the concentration of enzyme cleaving the given enkephalin analog conjugate in different areas of the brain.

Table 4-9 Reproducibility of Dissection Procedure

Brain region	Mean weight mg ± S.D.
Cerebellum	275 ± 25
Medulla oblongata	270 ± 32
Hypothalamus	112 ± 12
Striatum	331 ± 25
Midbrain	320 ± 23
Cortex	748 ± 26

Mean weights for various brain regions dissected from three animals ± S.D.

Table 4-10 Half-lives of enkephalin analog conjugate with double proline (T^+-Pro-Pro-DADLE) in different sections of rat brain (20% homoganate, pH7.4, 37^{0}C)

Different sections of Brain	Rate Constant (min^{-1})	Half-life ($t_{1/2}$) (min)
Cortex	0.16	4.3
Med. Oblongata	0.0097	71.8
Cerebellum	0.74	9.4
Striutum	0.056	12.3
Mid brain	0.056	12.3
Hypothalamus	0.024	28.5
Whole brain	0.059	11.66

Metabolic release of precursor peptide from conjugates containg progenitor sequence of TRH analogs

So far, these studies have concentrated on studying the sequence of enzymatic processes that results in the formation of the TRH analog or its immediate precursor in the CNS.

Stability studies of selected compounds have been performed in phosphate buffer, plasma and 20 % (w/w) brain and liver homogenate. Approximately 50 nmole of T^+-S-Gln-Leu/Nva-Pro-Gly-OH dissolved in water was added to 1 ml of rat plasma, brain or liver homogenate (20 %, w/w, in pH 7.4 Tris buffer), respectively, and the mixture was incubated at 37^0C in a temperature-controlled, shaking water bath. In a similar fashion, degradation of T^+-S-Gln-Leu-Pro-NH_2 has been studied in brain homogenate.

Incubation of the targetor-peptide conjugate with an already developed carboxamide at the C-terminus in rat brain homogenate showed that, depending on the spacer in question, a detectable to substantial amount of the compound may be cleaved at the spacer (Pro- or Ala)-glutaminyl (Gln) peptide bond. Figure 4-17 shows a representative ESI mass spectrum for the in vitro metabolism of T^+-Pro-Pro-Gln-Leu-Pro-NH_2 (m/z 670), where prolyl endopeptidase (EC 3.4.21.26) was the apparent processing enzyme. Indeed, the appearance of T^+-Pro-Pro-OH and the immediate precursor of the TRH analogue, Gln-Leu-Pro-NH_2 (m/z 355), is clearly shown. Conversion of Gln to pGlu by glutamyinyl cyclase, the final step in

the sequencial metabolism, has been well-documented for neuropeptides (Jackson, 1989, Fischer and Speiss, 1987). It was also shown in the related in vitro experiment that this conversion took place rapid (Table 4-11) and at a high yield (70%).

It is apparent that the designed sequence of metabolic activation should be valid, because there is a difference between the rate of processing by PAM (fast) and the rate of cleavage after the spacer (slower) (Table 4-11).

In summary, the peptidase cleavages were found to yield the target peptides from the brain-targeted precursors for both the DADLE- and TRH-CDSs, but the rates of reaction were varied in wide range depending on the prevailing peptidase and on the substrate properties (i.e., changing the composition and sequence of the amino acid residues within the spacer). Our hypothesis is that the reaction rate in the step involving the peptide release influences the pharmacological action according to a U-shaped curve; rapid cleavage could shorten the duration of action significantly, and slow release at the site of action could result in a peptide concentration below the therapeutic levels.

Table 4-11 CNS-stability of T^+-(S)-Peptide conjugates containing the progenitor (QXPG) and partially processed (QXP-NH_2) sequences

Peptide	Stability in brain-homogenate		Major metabolites
	$t_{1/2}$ (min)	k	
pGlu-LP-NH_2 [2]	58	0.012	QLP-NH_2.
pGlu-NvaP-NH_2 [2]	45	0.015	QLP-NH_2.
T^+-PP-QNvaPG-OH [2]	11	0.063	N.D.
T^+-PP-QLP-NH_2 [2]	48	0.014	QLP-NH_2
T^+-P-QLP-NH_2 [2]	51	0.013	QLP-NH_2
QLP-NH_2 [2]	0.77	0.9	pGlu-LP-NH_2

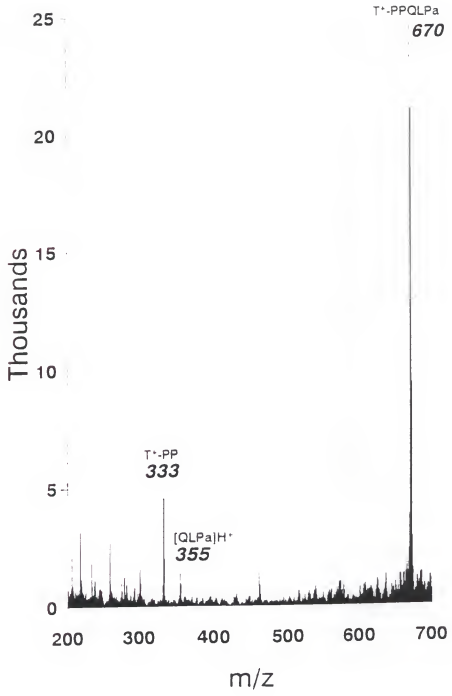

Fig. 4-17 ESI mass spectrum showing the release of TRH analog from the peptide conjugate (T^+-Pro-Pro-Gln-Leu-Pro-NH_2) (m/z 670) in vitro (rat brain homogenate, 37^0C, 1 hour incubation)

CHAPTER 5

SUMMARY AND CONCLUSIONS

During the completion of the studies, correlation of metabolic stability of the peptide precursors and the rate of peptide were investigated by in vivo and in vitro. It was also demonstrated that CNS-effect of a peptide after its targeting was possibly controlled in the CDS approach by the spacer manipulation.

In evaluation of the in vitro approach, studies of peptide degradation in brain homogenate carry out with peptide concentration from 10 to 100 μM in brain homogenate. Metabolism of synthetic dynorphins have been studied. The metabolite were buried in the chromatographic background coming from the brain homogenate. The in vitro approach was suitable for measuring the stability and obtaining kinetic data (k, $t_{1/2}$). However, detection of a full set of metabolites formed during degradation could not be done for most of the peptides studied.

In vivo approach for studying peptide metabolism in the brain has not been thoroughly evaluated. In vivo studies, microdialysis conditions has been set for delivering the required amount of peptide to the tissue and recovering the metabolites.

The two principal parameters has been adjusted for the experiments metabolites which are the concentration of peptide in the perfusion fluid and the

perfusion flow rate. The large peptide concentrations (1-5 mM) in the perfusion fluids and the flow rate of 0.8 μl/min appears to be the optimal for natural enkephalin. However, in vivo microdialysis combined with ESI mass spectrometry was not only suitable to find out whether the peptide was released from the trigonellyl compound but also to find some other metabolites.

HPLC profiles of T^+-[Pro-Pro^0]-[D-Ala^2]-[D-Leu^5]-enkephalin showed the appearance of [D-Ala^2]-[D-Leu^5]-enkephalin as the major metabolite. Biostability is a key issue in peptide drug development. For centrally active peptides, degradation in brain tissue takes on special significance. The enzymatic degradation rates of lanthionine-sandostatin and sandostatin, as reference material, have been determined.

In vivo microdialysis combined with MALDI and ESI mass spectrometry was used for metabolic optimization of brain-targeting for leucine-enkephalin analogs and thyrotropin releasing hormone analogs.

The in vitro and in vivo experiment showed agreement in terms of the major metabolite identified by HPLC retention times.

For conjugates with spacers intended to be cleaved by prolyl endopeptidase (PP, PA, AP and AA), half-lives were one to two orders of magnitude shorter than that of the dipeptidyl peptidase degradation.

Distribution of prolyl endopeptidase activity within the brain was investigted. In metabolites incubated with 100 μM of T^+-Pro-Pro-DADLE, the rate of reaction in medulla oblongata area were about 18 times slower than that in cortex area. These

results indicate the unequal regional distribution of PPCE (post-prolyl cleaving enzyme) activity against T^{+}-Pro-Pro-DADLE which results in the formation of DADLE, an opioid peptide analog.

The in vitro approach has been satisfactory for studying the degradation kinetics of synthetic dynorphins. The value of this approach is that biological stability of dynorphins can be compared, although homogenates may also contain many cytosolic peptidases to which, in vivo, dynorphins may not be exposed to.

In a study of prolyl endopeptidase activity within the brain, the results obtained in this study confirm and extend the previous findings in the whole brain which suggested that there were considerable variations in the concentration of enzyme for enkephalin analog conjugate in the different areas of the brain.

In conclusion, correlation of metabolic stability of the peptide precursors and the rate of peptide release with the observed pharmacological activities during the completion of the studies was done by in vivo microdialysis combined with LC/ESI-MS proven to be a successful technique for the elucidation of extracellular metabolism involving highly cationic peptides, such as dynorphins, in the brain.

APPENDIX

DATA FOR HPLC APPROACH

T^+-Pro-Pro-DADLE Data
Brain Metabolism ; In vitro calibration
C = F*Area + B

Equation: $y = 4*10^{-7}x - 4.3112$,

$r^2 = 0.9722$

time (min)	Conc (μM)	Area
0	11.0	39784320
3	8.4	34696768
6	9.5	39543008
10	8.2	33422640
20	5.9	24385856
30	4.0	16327072
45	2.8	12547000
70	0.6	2897170
110	0	0
170	0	0

Table A-1 Determination of AUC Using the Trapezoidal Rule for the following of T^+-Pro-Pro-DADLE Concentration-Time Data

Sample (n)	Time (min)	T^+-Pro-Pro-DADLE Concentration (μM)	AUC/t_0t_{n-1}(μM x min)
1	0	11.0	1/2(11.0 + 9.5)(6 - 0) = 61.5
2	6	9.5	1/2(9.5 + 8.2)(10 - 0) = 88.5
3	10	8.2	1/2(8.2 + 5.9)(20 -10) = 70.5
4	20	5.9	1/2(5.9 + 5.1)(30 - 20) = 55.0
5	30	4.0	1/2(4.0 + 2.8)(45 - 30) = 51.0
6	45	2.8	1/2(2.8+ 0.8)(70 - 45) = 45.0
7	70	0.6	1/2(0.6 + 0)(110 - 70) = 12.0
			AUC_{pep} = 383.5

Table A-2 Determination of AUC Using the Trapezoidal Rule for the following of DADLE as a Metabolite Concentration-Time Data

Sample (n)	Time (min)	DADLE Concentration (μM)	AUC/t_nt_{n-1}(μM x min)
1	0	0	1/2(0 +0.8)(6 - 0) = 2.4
2	6	0.8	1/2(0.8 + 1.5)(10 - 0) = 11.5
3	10	1.5	1/2(1.5 + 2.6)(20 -10) = 20.5
4	20	2.6	1/2(2.6 + 3.9)(30 - 20) = 32.5
5	30	3.9	1/2(3.9 + 3.4)(45 - 30) = 54.8
6	45	3.4	1/2(3.4 + 2.9)(70 - 45) = 78.8
7	70	2.9	1/2(2.9 + 1.1)(110-70) = 80.0
8	110	1.1	1/2(1.1 + 0)(170 - 70) = 55.0
			AUC_{met}= 335.5

$AUC_{met}/AUC_{pep}*100 = F_{AUC}$

335.5 / 383.5 *100 = 87.5 % for T^+-Pro-Pro-DADLE and its metabolite

T^{+}-Ala-Pro-DADLE Data
Brain Metabolism ; In vitro calibrarion
C = F*Area + B

Equation: $y = 2*10^{-5}x - 6.7427$,

$r^2 = 0.989$

time (min)	Conc (μM)	Area
0	63.4	4530042
20	34.8	2713872
40	23.2	1868459
60	14.9	1454792
120	0	0
240	0	0

Table A-3 Determination of AUC Using the Trapezoidal Rule for the following of T^{+}-Ala-Pro-DADLE Concentration-Time Data

Sample (n)	Time (min)	T^{+}-Ala-Pro-DADLE Concentration (μM)	$AUC/t_n t_{n-1}$ (μM x min)
1	0	63.4	1/2(63.4 + 34.8)(20 - 0) = 982
2	20	34.8	1/2(34.8 + 23.2)(40 - 20) = 580
3	40	23.2	1/2(23.2 + 14.9)(60 -40) = 381
4	60	14.9	1/2(14.9+ 0)(120 - 60) =447
			AUC_{pop} = 2390

Table A-4 Determination of AUC Using the Trapezoidal Rule for the following of DADLE as a Metabolite Concentration-Time Data

Sample (n)	Time (min)	DADLE Concentration (μM)	AUC/t_nt_{n-1}(μM x min)
1	0	0	1/2(0 +8.9)(20 - 0) = 89
2	20	8.9	1/2(8.9 + 11.9)(40 - 20) = 208
3	40	11.9	1/2(11.9 + 8.3)(60 -40) = 202
4	60	8.3	1/2(8.3 + 6.8)(120 - 60) = 453
5	120	6.8	1/2(6.8 + 4.3)(240 -120)= 666
6	240	4.3	1/2(4.3 + 0)(480 - 120) = 516
			AUC_{met}= 2134

$AUC_{met}/AUC_{pep}*100 = F_{AUC}$

2134 / 2390 *100 = 89.3 % for T^+-Ala-Pro-DADLE and its metabolite

Glu-Leu-ProNH_2 Data
Brain Metabolism ; In vitro calibrarion
C = F*Area + B

Equation: $y = 4*10^{-6}x - 1.0718$,

$r^2 = 0.9908$

time (min)	Conc (μM)	Area
0	6.1	2251845
1	3.3	1487804
2	1.2	442572
5	0.07	61214
10	0	0

Table A-5 Determination of AUC Using the Trapezoidal Rule for the following of Glu-Leu-$ProNH_2$ Concentration-Time Data

Sample (n)	Time (min)	Glu-Leu-$ProNH_2$ Concentration (μM)	AUC/t_nt_{n-1}(μM x min)
1	0	6.1	1/2(6.1 + 3.3)(1 - 0) = 4.7
2	1	3.3	1/2(3.3 + 1.2)(2 - 1) =2.25
3	2	1.2	1/2(1.2 +0.07)(5- 2) =1.95
4	5	0.07	1/2(0.07 + 0)(10 -5) =0.18
			AUC_{pep}= 8.918

Table A-6 Determination of AUC Using the Trapezoidal Rule for the following of pGlu-Leu-ProNH_2 as a Metabolite Concentration-Time Data

Sample (n)	Time (min)	pGlu-Leu-ProNH_2 Concentration (μM)	$AUC/t_n t_{n-1}$(μM x min)
1	0	0	1/2(0+ 0.076)(1 - 0) = 0.036
2	1	0.076	1/2(0.076 + 0.93)(2 - 1) = 0.53
3	2	0.93	1/2(0.93 +1.07)(5- 2) = 3.00
4	5	1.07	1/2(1.07 + 0)(10 -5) = 2.68
			AUC_{met} = 6.246

$AUC_{met}/AUC_{pep}*100 = F_{AUC}$

6.246/ 8.918 *100 = 70.0 % for T^c-Pro-Pro-DADLE and its metabolite

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Associate Professor of Pharmaceutics

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